

**Purification of Human DNA for DNA Profiling from Body
Fluid Contaminated Soil using Superparamagnetic Iron
Oxide Nanoparticles (Spions)**

by

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Abstract

Extraction of good quality DNA from soil samples is difficult due to the co-extraction of humic acid and phenolic compounds which can inhibit downstream DNA profiling of samples. Several methods are available for DNA extraction from soil including a number of commercially available kits. We used varying amounts (5 mg to 250 mg) of soil contaminated with human body fluid (saliva, semen and blood) to compare commercial kits (Powersoil® and PrepFiler™), a standard phenol/chloroform extraction method, and a method employing superparamagnetic iron oxide nanoparticles (SPIONs) generated in-house. Extraction success and level of purity of the human DNA extracted using these methods were assessed by quantifying the amount of DNA recovered using a Nanodrop 2000 and the amount of humic acid recovered using UV-Vis spectroscopy. Furthermore, successful human DNA profiling was assessed using an in-house generated four-plex PCR assay amplifying fragments of 70 bp, 194 bp, 305 bp and 384 bp, as well as the commercial Powerplex® 16 DNA profiling kit amplifying 16 different amplicons. Our results indicate that the PrepFiler™ kit and phenol/chloroform whilst successful at extracting DNA, contained very high levels of humic acid in the extracted DNA greatly impairing DNA profiling even after dilution of DNA (1 in 10) and addition of higher amounts of *Taq* (5U). The PowerSoil® kit in contrast, performed best by recovering the most DNA whilst removing the most humic acid, and good quality DNA profiles were obtained without the requirement for dilution of the DNA or addition of extra *Taq*. However, using extensive optimisation procedures using SPIONs, we have developed a protocol that is able to achieve results very similar to those achieved using Powersoil®, thereby greatly reducing the cost per sample.

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Abbreviations

ABS- Aqueous Biphasic Systems

BSA- Bovine Serum Albumin

Ca²⁺- Calcium ions

CTAB- Cetyltrimethyl Ammonium Bromide

DNA- Deoxyribonucleic Acid

DTT- Dithiothreitol

EDTA- Ethylenediaminetetraacetic Acid

HA- Humic Acid

HMW- High Molecular Weight

MLB- Mean Local Balance

mtDNA- Mitochondrial Deoxyribonucleic Acid

Na⁺- Sodium ions

NaCl- Sodium Chloride

PEG- Polyethylene Glycol

PVP- Polyvinylpyrrolidone

qPCR- Quantitative Polymerase Chain Reaction

SDS- Sodium Dodecyl Sulfate

SNPs- Single Nucleotide Polymorphism

SPIONs- Superparamagnetic iron oxide nanoparticles

STR- Short Tandem Repeats

TAE- Tris-acetate-EDTA

T_m- Melting Temperature

TPH- Total Peak Height

Tris- Tris(hydroxymethyl)aminomethane

1. Introduction

1.1. A brief history of forensic DNA analysis

Forensic science aims to help judges and juries in solving legal issues, not only in criminal but also in civil cases. The main aspects of forensic science are the identification, individualisation and classification of physical evidence found at a crime scene.

The ultimate goal of forensic science, in the identification process, is individualisation which gives information on the origin of a particular piece of evidence. For many years only few types of evidence, such as fingerprints, could be unequivocally individualized. All other types of evidence could only be said to be consistent with originating from a particular location or individual. However, in 1980s, one particular biological tool revolutionised forensic investigation – DNA analysis.

Any type of organism can be identified by examination of its DNA sequences which is unique to that organism. Every cell carries a copy of the DNA and, all DNA exhibits variability both among and between species; therefore, any biological material associated with a legal case carries information about its source that can be used to distinguish between individuals since the order of base pairs in the DNA of every individual is different except in identical twins.

Forensic DNA analysis has greatly evolved since it was first used over 30 years ago and has become an indispensable and routine part of forensic casework. Employing extremely sensitive PCR-based techniques to analyse biological material has enabled forensic scientists to link suspects to crime scenes or one crime scene to another using biological evidence left behind in form of saliva, hair, skin cells or various body fluids.

The aim of forensic DNA analysis is to identify with as much certainty as possible the origin of particular evidence. Many databases, tests and techniques that are currently being widely and routinely used in forensic investigations have been developed and refined in a process which, for some disciplines, had taken centuries and for more recent technologies, decades.

The concept of using DNA as a method of individualisation emerged in 1985 when Alec Jeffreys discovered a significant variation between individuals in particular regions of DNA (Gill *et al.* 1985, Jeffreys Alec J. *et al.* 1985a, Jeffreys A. J. *et al.* 1985b). Jeffreys found that certain regions of DNA contained a very characteristic pattern of short sequences repeated in a tandem fashion. He also discovered that the number of repeats could vary between individuals (and within for heterozygous loci) - these regions of DNA became known as Variable Number Tandem Repeats (VNTR) or Minisatellites. Upon analysis using Southern blotting, the unique patterns of the polymorphic regions in an individual and similarities in principle to fingerprinting methods for identification resulted in the use of the term DNA fingerprinting (Jeffreys *et al.* 1992).

Despite the fact that using minisatellites resulted in successful individualisation, the technique was time consuming and laborious and more importantly, required large amounts of well-preserved DNA which usually is almost impossible to acquire from forensic samples. Due to these limitations, DNA fingerprinting was subsequently replaced by STR DNA profiling.

Short tandem repeats (STRs) or microsatellites are relatively short (100 bp to 350 bp) regions which consist of tandemly repeating units of 2-6 bp that can be repeated many times within a particular locus (Queller *et al.* 1993).

The microsatellites in use today for human DNA profiling are all tetra- or penta-nucleotide repeats because dinucleotide repeats tend to suffer from artifacts such as PCR stutter (Butler 2005).

The very first widely used multiplex consisted of 4 STRs (quadruplex) (Kimpton *et al.* 1993). However, due to its very high match probability (1 in 10000), the first criminal case involving STRs was done in conjunction with single-locus probe (SLP) DNA fingerprinting (Jobling and Gill 2004). Subsequent addition of two more highly variable STRs reduced the match probability to 1 in 50 million, and this multiplex was then named “second-generation multiplex” (SGM). This multiplex also included the amelogenin marker thereby revealing the sex of a sample donor.

In 2000, an additional 4 loci were added to this multiplex, which was renamed SGM Plus, further reducing the match probability to 1 in 10^{13} (Cotton *et al.* 2000).

The ability to study multiple STR markers in parallel using multicolour fluorescence detection technologies has revolutionized STR DNA profiling, allowing large numbers of loci to be analysed simultaneously, giving very high levels of discrimination between individuals. Many different commercial STR multiplex kits are currently available (Table 1.) and the AmpFlSTR GlobalFiler kit (Applied Biosystems) can simultaneously amplify 24 different STR loci including amelogenin and Y-indel. Another commonly used, in forensic human DNA analysis, STR multiplex, PowerPlex®16 (Promega), uses four dye colours system to amplify and detect 16 different DNA targets. PowerPlex®16 is highly optimized and robust with an easy to follow protocol (PowerPlex®16 Manual, Promega). A complete profile can be generated within four hours after the DNA extraction procedure.

Table 1: Current, commercially available STR systems.

	SGM Plus®	Profiler Plus ®	Identifiler®	PowerPlexFusion	PowerPlex® 16	PowerPlex® 21	AmpFISTR GlobalFiler
	Amelogenin D16S359 D18S51 D19S433 D21S11 D2S1338 D3S1358 D8S1179 FGA TH01 vWA	Amelogenin D13S317 D18S51 D21S11 D3S1358 D5S818 D7S820 D8S1179 FGA vWA	Amelogenin CSF1PO D13S317 D16S359 D18S51 D19S433 D21S11 D2S1338 D3S1358 D5S818 D7S820 D8S1179 FGA TH01 TPOX vWA	Amelogenin CSF1PO D10S1248 D12S391 D13S317 D16S359 D18S51 D19S433 D1S1656 D21S11 D22S1045 D2S1338 D2S441 D3S1358 D5S818 D7S820 D8S1179 DYS391 FGA Penta D Penta E TH01 TPOX vWA	Amelogenin CSF1PO D13S317 D16S359 D18S51 D21S11 D3S1358 D5S818 D7S820 D8S1179 FGA Penta E PentaD TH01 TPOX vWA	Amelogenin CSF1PO D12S391 D13S317 D16S359 D18S51 D19S433 D1S1656 D21S11 D2S1338 D3S1358 D5S818 D6S1043 D7S820 D8S1179 FGA PentaD PentaE TH01 TPOX vWA	Amelogenin CSF1PO FGA D1S1656 D12S391 D2S441 D10S1248 D22S1045 TH01 TPOX vWA D3S1358 D5S818 D7S820 D8S1179 D13S317 D16S539 D18S51 D21S11 D2S1338 D19S433 SE33 DYS391 Y-indel
Probability of identity	7.04x10 ⁻¹⁴	6.11x10 ⁻¹²	6.18x10 ⁻¹⁹	6.58x10 ⁻²⁹	2.82x10 ⁻¹⁹	6.77x10 ⁻²⁷	7.73x10 ⁻²⁸

Multiplexes are analyzed using automated genetic analysers consisting of multi-channel capillary electrophoresis systems that are used to detect fluorescently labeled PCR products. As a result of such automation, STRs became ideal for forensic DNA profiling, greatly reducing costs and increasing throughput.

Furthermore, STR typing requires very little template DNA since it is PCR-based. Small quantities of poor quality DNA obtained during forensic investigations are often the only samples available, making the PCR technique highly useful (Decorte 2010, White *et al.* 1989).

Development of PCR-based DNA profiling not only allowed for the generation of profiles from types of samples that had not previously been examined such as cigarette butts

(Hochmeister *et al.* 1991), single human hairs (Higuchi *et al.* 1988), urine (Brinkmann *et al.* 1992), fingernail scrapings (Wiegand *et al.* 1993) and bite marks (Sweet *et al.* 1997), it also improved the success in generating useful profiles from old, burnt, degraded bone and tissue samples (Clayton *et al.* 1995, Parsons *et al.* 2007, Whitaker *et al.* 1995) given small STR amplicon sizes.

In cases where the DNA has become so degraded as to preclude STR amplification, a different type of genetic marker, single nucleotide polymorphisms (SNPs) is increasingly being used. SNPs are DNA sequence variations occurring when a single base pair in the genome is altered (Rocha Dominique *et al.* 2006). Due to its very small amplicon size, lower mutation rate in comparison to the STRs as well as abundance in the human genome, SNPs are recent markers of interest in the field of forensic science (Budowle and van Daal 2008, Kim *et al.* 2010). However, due to limitations such as lack of widely established SNP databases, and requirement of large multiplexing assays due to the lower power of discrimination offered by SNPs given their biallelic nature, SNPs are unlikely to replace the currently used STRs in the near future (Jobling and Gill 2004).

1.2. Polymerase Chain Reaction (PCR) and PCR inhibition

The polymerase chain reaction (PCR) discovered in 1983 by Kary Mullis, is now an indispensable technique employed by medical and biological research laboratories for a variety of applications. While DNA fingerprinting required the input of significant amounts of DNA, PCR based STR profiling requires as little as 0.06 ng of DNA template (Ensenberger *et al.* 2010).

Environmentally challenged biological samples found at a crime scene can produce numerous problems in human DNA profiling including allele drop-out, peak imbalance and loss of signal (as a result of poor amplification efficiency).

For many years forensic geneticists have looked into methodological issues associated with degraded DNA (Alaeddini *et al.* 2010), tissue preservation (Martínez *et al.* 1994) and strategies to repair degraded DNA (Vasan *et al.* 1996). One less explored topic is the co-extraction of PCR inhibitors along with DNA.

PCR inhibition is the most common cause of PCR failure when sufficient amount of DNA template is present in the reaction. While the co-existence of PCR inhibitors is well known and a number of methods to improve PCR amplification have already been developed (Bourke *et al.* 1999, Moreira 1998, Yang *et al.* 1998) (Table 2), the mechanism of the inhibition is often unclear.

According to studies by (Huggett *et al.* 2008, Opel *et al.* 2010), co-purified PCR inhibitors affect different PCR products differently. Opel *et al.* (2010) found that primers with higher melting temperature were usually less affected by inhibition. This suggested that the amplicon/primer sequence may have a direct effect on PCR inhibition, where- primers with higher melting temperature due to their strong binding to the DNA template may prevent the binding of inhibitors (Opel *et al.* 2010).

Table 2: Common inhibitory substances and their mode of inhibition encountered during DNA profiling as well as methods of overcoming PCR inhibition.

Substrate(s)	Inhibitor(s)	Mode of inhibition	Facilitator(s)	Reference
Faeces and plants	Bile salts and complex polysaccharides	Interaction with DNA template (sequestration of DNA)	BSA, gp32, sample dilution	(Rouhibakhsh <i>et al.</i> 2008) (Radstrom <i>et al.</i> 2004)
Bones and connective tissues	Collagen	Binds to DNA template	Sample purification, use of less sensitive <i>Taq</i> polymerases, addition of Mg^{2+}	(Burkhart <i>et al.</i> 2002) (Opel <i>et al.</i> 2010)
Bones	Calcium ions	Competitive inhibitor of Mg^{2+} required for <i>Taq</i> activity	Sample dilution, chelation, addition of Mg^{2+}	(Opel <i>et al.</i> 2010) (Bickley <i>et al.</i> 1996)
Clothing dyes (e.g. indigo)	Dyes	Affects DNA template by incorporating into DNA structure.	Sample purification,	(Larkin and Harbison 1999)
Lactoferrin and haemoglobin(Blood)	Iron ions	Competitive inhibitor of Mg^{2+} required for <i>Taq</i> activity	Sample dilution, chelation, addition of Mg^{2+}	(Radstrom <i>et al.</i> 2004)
Blood	Heme	Binds to <i>Taq</i> polymerases causing dissociation of the DNA-polymerase complex	BSA, gp32	(Kreader 1996) (Akane <i>et al.</i> 1994)
Hair and skin	Melanin	Binds to DNA	Sample purification, Sample dilution	(Opel <i>et al.</i> 2010) (Eckhart <i>et al.</i> 2000)
Soils and bones	Millard Products	DNA trapped in complex polysaccharide-rich matrix (inaccessible to <i>Taq</i> polymerases)	Sample purification (repeated silica extraction)	(Alaeddini 2012)
Environmental samples containing soil	Phenolic compounds (e.g. humic, fulvic and tannic acids)	Chelating with Mg^{2+} , Humic acids have also been reported to directly affect <i>Taq</i> polymerases and DNA through sequence specific binding of DNA, reducing the amount of amplifiable template	Retardation of phenolic migration in PVP-containing agarose gel electrophoresis, Sample dilution, Addition of Mg^{2+} , Ion-exchange chromatography,	(Mayer and Palmer 1996) (Herrick <i>et al.</i> 1993) (Tebbe and Vahjen 1993) (Tsai and Olson 1992a)
Semen swabs from sexual assaults, Microorganisms found in environmental samples	Vaginal microorganisms, Non-target DNA	DNA sequestration, Reduction of primer concentration by non-specific binding to non-target DNA molecules	Sample dilution, Gel filtration,	(Lienert and Fowler 1992)
Urine	Urea	Denaturation of <i>Taq</i> polymerases	Sample dilution, addition of <i>Taq</i>	(Abu Al-Soud and Radstrom 1998)

Although the inhibition is thought to be the result of many factors e.g. direct binding of the inhibitors to the DNA polymerase; interaction of the inhibitors with the DNA polymerase during the primer extension step or binding of the inhibitors to the DNA template, research has been mostly focused on the inhibition of the function of the polymerase (Opel *et al.* 2010).

Different inhibitors have different effects on a PCR. Collagen and Ca^{2+} ions are components of connective tissue and bone and very often co-extracted during DNA extraction from skeletal remains. Although the mode of inhibition differs between those two inhibitors (Opel *et al.* 2010), they both can cause a reduction in PCR efficiency or even PCR failure when the concentration of the inhibitors is too great ($>8 \mu\text{g}$) to be overcome by the polymerase activity. In addition, like all *Taq* inhibitors, Ca^{2+} and collagen affect the PCR efficiency equally, regardless of the length of amplicons or the melting temperature of primers used (Opel *et al.* 2010).

Melanin is a pigment found in skin and hair, and is a possible inhibitor present in telogen hair samples (Yoshii *et al.* 1993). Unlike collagen, melanin affects PCR by binding to the DNA template therefore having a greater effect on larger amplicons. Opel *et al.* (2010) found that the presence of melanin in the PCR had no or a very small effect on 100 bp amplicons whereas complete loss of signal was observed with amplicons longer than 400 bp.

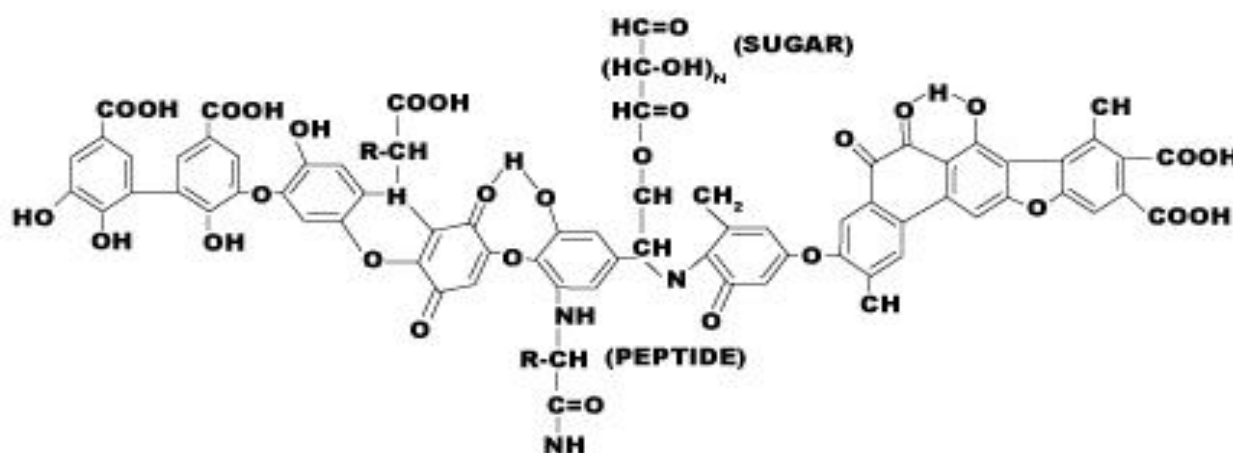


Figure 1: Example of a typical humic acid molecule containing a variety of different functional groups such as carboxyl and phenolic groups. Structure of humic acids molecules can vary and often involves crosslinking with sugars and peptides.

In addition, a higher concentration (4 $\mu\text{g/mL}$) of the inhibitor was required to significantly reduce the PCR efficiency with the primer sets with higher T_m .

However, not all inhibitory substances encountered during forensic case work exhibit a such straight forward mode of inhibition.

Humic substances (HS) are very well known and documented cause of PCR failure in forensic and microbial studies.

HS can be subdivided into three main subgroups: Humus, Humic (HA) and Fulvic (FA) acids where HA comprise one of the major fractions of humic substances (Saulnier and Andremon 1992).

Humic substances are amorphous, dark-coloured and highly stable organic macromolecules (Figure 1) found in soils and produced during decay processes through the Maillard reaction (Rocha Julio Cesar *et al.* 1998). Depending on the type of soil, humic substances can make up 5.0-7.63 $\mu\text{g/mg}$ of soil (Tebbe and Vahjen 1993) and approximately 700-3300 $\mu\text{g/mL}$ of humic and humic-like compounds (e.g. fulvic acid) can be recovered during DNA extraction from soil samples (Alaeddini 2012).

Due to the charge to mass ratio, their structural heterogeneity, high molecular weight and their similarities to DNA, humic compounds feature the most problematic impurity in DNA extraction procedures (Zipper *et al.* 2003).

HAs have physiochemical properties similar to those of the phosphate groups in the DNA backbone (Dong *et al.* 2006). The high number of hydroxyl and carboxyl groups gives the HAs a negative charge similar to DNA which therefore can compete with DNA for the binding sites during purification steps (silica based DNA extraction) (Roose-Amsaleg *et al.* 2001). HAs are thought to chelate the magnesium ions required for the *Taq* polymerase activity (Tsai and Olson 1992a). It has also been reported that free DNA in soil rapidly adsorbs and binds HAs (Crecchio and Stotzky 1998, Tsai and Olson 1992b) which provides protection against nucleases (Alvarez *et al.* 1998) but entraps the DNA making it inaccessible to the *Taq* polymerase. Furthermore, it has been reported that, like all other oligomeric compounds with free phenolic groups (e.g. tannins), HAs can covalently bind to and inactivate polymerases (Young *et al.* 1993).

As little as 0.08 µg/mL of HA has been found to be sufficient to inhibit PCR amplification and 0.5 µg/mL will inhibit restriction enzymes (Tebbe and Vahjen 1993) where the level of inhibition caused by the HAs is directly associated with the amount of the inhibitor present in a PCR reaction. In a different study complete inhibition of certain PCR reactions has been shown to occur with 0.05 µg/mL of HA (Tien *et al.* 1999).

Studies on inhibition from HAs and melanin indicated a sequence specific manner of PCR inhibition through binding to and inactivating a portion of available DNA template (Opel *et al.* 2010). However, the exact mode of inhibition is unclear since different studies suggest either *Taq* polymerases or DNA templates to be the targets for binding (Table 2).

1.3. Overcoming PCR inhibition

Procedures that overcome PCR inhibition need to be developed if PCR is to be successfully used for the analysis of environmental samples. Following DNA extraction and detection of PCR inhibition, some laboratories employ routine inhibitor trouble-shooting strategies such as: sample dilution (Alonso *et al.* 2001), use of BSA or 32 T4 protein (Oikarinen *et al.* 2009), heat soaked PCR (Ruano *et al.* 1992), hot start PCR (Kermekchiev *et al.* 2003) and addition of extra polymerase enzymes (Eilert and Foran 2009).

1.3.1. Sample dilution

The most common technique used to overcome PCR inhibition is diluting the DNA extract sufficiently to eliminate the inhibition (Imaizumi *et al.* 2005). However, this technique although effective especially in case of mtDNA or microbial DNA analysis where high copy number DNA is available, may not be the best choice in case of highly degraded DNA templates where the copy number is usually already very low (Taberlet *et al.* 1996).

1.3.2. Amplification facilitators

The addition of PCR facilitators has been found to improve the specificity of PCR (Jensen *et al.* 2010, Sarkar *et al.* 1990, Wu and Yeh 1973) (Table 2).

The possible mode of action of protein-based facilitators such as bovine serum albumin (BSA) or single stranded DNA binding T4 gene 32 protein (gp32) (Kreader 1996), is thought to lie in their capacity to bind certain inhibitors and therefore inactivate them (Abu Al-Soud and Radstrom 2000).

With increased concentration of the facilitators, the number of free inhibitors present in the reaction will be lowered and therefore indirectly increase the PCR efficiency. In addition, BSA cannot relieve inhibition caused by agents interacting with the DNA template only, such as collagen (Alaeddini 2012).

Betaine has been suggested to increase PCR specificity and product yield (Jensen *et al.* 2010) as well as the thermal stability of proteins (Santoro *et al.* 1992). Due to both positive and negative charges present on the betaine molecules at pH close to neutral, the PCR facilitator activity has been suggested to lie in its ability to destabilize GC-rich DNA sequences (Rees *et al.* 1993).

The presence of polysaccharides in a DNA preparation can inhibit PCR. Cetyltrimethyl ammonium bromide (CTAB) is a surfactant useful for isolation of DNA from tissues containing high amounts of polysaccharides. Under the high-salt conditions the CTAB binds the polysaccharides, removing them from the solution (Alaeddini 2012).

1.3.3. Addition of higher amounts/use of different types of polymerases

The addition of extra polymerase enzyme may overcome inhibition caused by inhibitors targeting that particular enzyme. However, increased concentration of polymerase may result in higher frequency of non-specific amplification as well as greatly raise the cost of each amplification reaction (Abu Al-Soud and Radstrom 2000).

Different polymerase enzymes exhibit different properties in regards to their sensitivity to inhibitors. Hot-start enzymes such as Ex *Taq* HS, have been found to be resistant to a much higher concentration of inhibitors (such as calcium ions or collagen) from skeletal remains (Eilert and Foran 2009). Some DNA polymerases (e.g. *Pfu*, Act-X-Short) have also been found to display greater tolerance to different inhibitors including HA molecules (Eilert and Foran 2009). Genetically modified polymerases (e.g. Klentaq 1) have shown to be highly resistant to high concentration of variety of inhibitors (Kermekchiev *et al.* 2009) but other studies have suggested that the enzyme that is least affected by inhibition coming from HAs is the *Pfu* DNA polymerase (Matheson *et al.* 2010).

1.4. DNA Extraction from soil

Soils are very complex and heterogenous matrices, composed of minerals, and organic matter, differing in texture, colour, structure and chemical and biological composition (Mitchell and Soga 1976). Depending on the size of soil particles, soils can be divided into three main soil types: sands, silts and clay. However, in nature, soils usually occur as a mixture of the three (loams), where the contribution of each type varies (e.g. silty-loams – mainly silt with lower amounts of clay and sand). Among the mentioned soil types, clay has the highest organic matter content followed by silts and sands. Sands are usually very low in organic matter due to the lack of sufficient waterlogging allowing plant growth. Furthermore, silty type of soils (silty-loams) are the most commonly used in agriculture and gardening since their waterlogging and nutrients capacity is optimal for a wide range of different plants (Mitchell and Soga 1976). In addition, unlike clay, silty-loams shows much faster rate of organic matter decomposition resulting in much higher nutrients availability as opposed to clays or sands making them ideal for agriculturing (Donahue *et al.* 1977).

The quality of DNA extracted is of utmost importance as the goal is to obtain a reliable STR profile. Therefore, extraction methods that enable forensic analysts to isolate DNA from samples that contain small quantities of biological material, obtain DNA at a high concentration so that the volume of extract used for PCR is minimal and remove all possible PCR inhibitors or substances that could potentially interfere with the amplification are highly desirable.

Extraction of good quality DNA from soil is particularly difficult due to the co-extraction of HA and phenolic compounds which inhibit PCR (Alaeddini 2012, Hudlow *et al.* 2008, Zahra *et al.* 2011).

Isolation of DNA involves cell lysis and DNA purification steps to remove unwanted cellular material and other substances such as inhibitors present in the lysate while recovering high quality DNA. A variety of lysis methods are available including physical disruption, chemical lysis and enzymatic lysis. Freeze-thawing and bead beating methods of physical disruption are most commonly used as they yield the largest amounts of DNA (Delmont *et al.* 2011). However the major drawbacks of these techniques are that large concentrations of humic compounds are also recovered and shearing of DNA molecules may occur (Simonet *et al.* 1991). Chemical lysis procedures vary but usually include mixtures containing detergent (e.g. sodium dodecyl sulphate) in the presence of NaCl and buffers such as Tris or phosphate as well as chelating agents such as EDTA to inhibit nucleases and disperse soil particles (Moreau *et al.* 1999). Enzymatic lysis using various enzymes such as proteinase K, protease or lysozyme that promote cell lysis and digest proteins bound to DNA molecules is also used. For samples containing large amounts of inhibitory substances, PVP containing agarose gel

Table 3: Current commercially available DNA isolation kits.

DNA extraction kit	Recommended sample amount [mg]	Cell lysis	DNA binding	Ref.
PowerSoil®	250	Bead-beating	Silica spin columns	(Makhalanyane <i>et al.</i> 2013)
PrepFiler™ Forensic DNA isolation kit	50	Chemical lysis	Magnetic beads	(Brevnov <i>et al.</i> 2009)
Phenol/Chloroform organic DNA extraction	Up to 5000	Chemical lysis	Ethanol precipitation	(Steffan <i>et al.</i> 1988)
QIAamp Stool	220	Chemical lysis	Silica spin columns	(Jain <i>et al.</i> 2012)
UltraClean®	250-1000	Bead-Beating	Silica spin columns	(Claassen <i>et al.</i> 2013)

electrophoresis, Sephadex G-200 chromatography, PEG two-phase extraction have been used as an additional step of DNA extraction in order to separate DNA from PCR inhibitor (Rahimpour *et al.* 2006).

More recently a number of different DNA extraction kits have been developed for isolation of DNA from problematic samples such as soils or faeces (Table 3). However, the main principle behind the DNA isolation procedure (i.e. removal of inhibitors) using currently available commercial kits and older purification techniques such as Phenol/Chloroform, has not changed.

Most commercial DNA extraction kits and published protocols utilise detergent to lyse cells as the initial step of DNA isolation. Second, to eliminate contaminating RNA and proteins, RNase and Proteinase K are added. In addition, some more sophisticated commercial kits use silica matrices or magnetic beads to avoid exposure to organic solvents such as chloroform (Table 3). Other methods selectively purify DNA from cellular debris by high salt concentration and ethanol precipitation followed by several 70 % ethanol washes.

By combining the DNA isolation and purification into one simple procedure, DNA extraction kits are generally much faster than the organic Phenol/Chloroform method especially in case of samples containing high amounts of inhibitors that are chemically similar to DNA inhibitors where the simple organic method fails to separate the two and usually require additional steps such as PVP-agarose gel electrophoresis or size-exchange chromatography.

However, most of the commercial kits available on the market rely on silica filter columns which involves a number of centrifugation and sample re-pipeting steps that are time-consuming, not suitable for automation and could potentially be the cause of contamination.

1.5. Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

The last few years have seen major developments in DNA extraction methods using different types of magnetic nanoparticles that can separate DNA from the rest of the lysate e.g. superparamagnetic iron oxide nanoparticles (SPIONs) (Berensmeier 2006, Sebastianelli *et al.* 2008) . SPIONs have been successfully used to extract DNA from soil (Sebastianelli *et al.* 2008) and commercial kits using SPIONs have been developed for use with soil (e.g. PrepFilerTM) (Brevnov *et al.* 2009).

In Sebastianelli *et al.*'s study, three DNA isolation methods were compared for soil samples—Phenol/Chloroform; a commercially available kit SoilMasterTM (Epicentre Biotechnologies), and SPIONs synthesised in-house. Their results suggested that SPIONs enabled DNA extraction to higher quantities than Phenol/Chloroform and SoilMasterTM methods. The SPION extracted DNA also appeared to be of better quality, allowing successful PCR amplification of a 566 bp bacterial 16S rDNA product in 3/3 samples, as opposed to 2/3 samples extracted using SoilMasterTM and 0/3 with Phenol/Chloroform extracted samples (suggesting presence of PCR inhibitors since HMW DNA was observed in the extracts). However, these experiments were limited in their scope since they relied on the amplification of a single product from a high copy number bacterial gene which does not appropriately reflect generation of a reliable DNA profile across several loci. Several others have also reported successful extraction of microbial DNA from soil (Dineen *et al.* 2010, Hurt *et al.* 2001, Jacobsen and Rasmussen 1992, Whitehouse and Hottel 2007) but because soil contains a huge amount of microbial DNA (10^9 cells/ g of soil - Sebastianelli *et al.* 2008), the purified DNA had to be first diluted in order to optimise the template concentration for PCR. However, such a practice also reduces the concentration of potential PCR inhibitors allowing

successful amplification. In addition, sample dilution might not be a suitable option when dealing with highly degraded or very low amounts of starting material.

Magnetic separation of nucleic acids for purification has several advantages over other techniques used for the same purpose- e.g using SPIONs nucleic acids can be extracted directly from crude samples including blood, tissue homogenates, water, soils etc. Furthermore, due to the possibility of adjusting the magnetic properties of the solid materials, the magnetic particles can be removed easily and selectively even from viscous suspensions. In fact, magnetic separation is the only feasible method that can be used for recovery of very small particles (i.e. silica beads) in the presence of variety of biological debris and other materials of similar size.

In addition, these relatively new separation techniques can also serve as a basis for automated low- and high-throughput procedures greatly reducing time and costs of each DNA extraction. Furthermore, centrifugation steps and the risk of cross-contamination when using traditional methods of DNA isolation can be eliminated.

1.5.1. SPIONs structure and DNA binding mechanism

The increasing use of magnetic particles as a solid phase (carrier) for DNA binding has many advantages compared to other non-magnetic separation techniques. The term “magnetic” means that the magnetic carrier obtains a magnetic moment when placed in either magnetic or electromagnetic field. Thus, it can be displaced regardless of the presence of any other non-magnetic material. In other words, particles having a magnetic moment can be removed from almost any mixture by simply applying a strong enough magnetic field, e.g. by using a permanent magnet. It is a quick, simple and very efficient way of separating the particles from

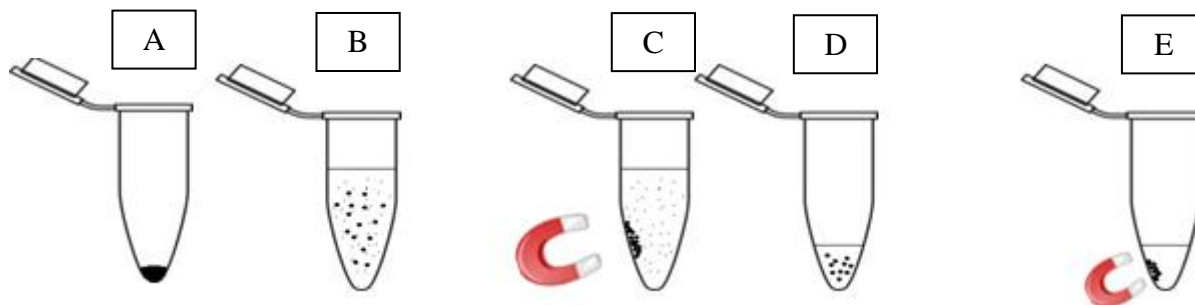


Figure 2: Schematic procedure for nucleic acid extraction using magnetic beads technology. Upon addition of an appropriate lysis buffer to a tube containing the sample (A) and carrying out the lysis, DNA molecules are released from cells into the liquid phase. After the addition of magnetic beads and an appropriate binding buffer, DNA binds to the surface of the beads (B). The magnetic particles are then separated from the rest of the lysate by placing it in a magnetic field (C) and the supernatant discarded. Figure 2 D and E shows the DNA elution steps where DNA is being released from the surface of the beads into the liquid phase upon addition of a salt-free elution buffer. Magnetic field allows separation of the magnetic particles from the buffer containing purified DNA molecules (E).

the lysate after the DNA binding step (Figure 2) where applying a magnet to the side of the tube containing the sample is usually sufficient enough for the separation to occur.

Magnetic carriers with affinity ligands (e.g. DNA probes) or those prepared using a variety of polymers with affinity for the target nucleic acid are used in the extraction processes (Spanova *et al.* 2006). Especially suited for this purposes are SPIONs, which do not interact with each other in the absence of the magnetic field. Thus, they do not form aggregates which would greatly reduce the active surface area for nucleic acid binding.

Many magnetic particles are commercially available but can also be relatively easily synthesized in the laboratory. Although a variety of different magnetic particles, including “naked” iron oxide, modified bacterial magnetites (Amagliani *et al.* 2006), magnetic capture hybridization (uses ssDNA probes to bind DNA), gelatin-, PEI/Au- (Sun *et al.* 2010), silica-coated magnetic particles (Sebastianelli *et al.* 2008) has been developed, and the basic structure and binding mechanism is shared among them.

SPIONs are produced in a two-step process involving Fe_3O_4 synthesis (reaction carried out using $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ under stream of nitrogen at 25°C followed by addition of urea (25% w/w) and incubation at 80°C for ~30 min) and silica coating step, where the

synthesized Fe_3O_4 particles are dispersed in a mixture containing ethanol, deionized water, urea and tetraethyl orthosilicate, and incubated at 40 °C for 12 h (Jiang *et al.* 2012).

The diameter and shape of the particles can vary ($\sim 0.5\text{-}20\ \mu\text{m}$) (Spanova *et al.* 2006). The most commonly used magnetic particles for DNA isolation are silica-coated magnetic nanoparticles. The structure of silica-coated magnetic particles is shown in Figure 3- each particle consists of a magnetisable core (Fe_3O_4) and a layer of silica coating which carries a negative charge when pH is around neutral (Berensmeier 2006). However, since both nucleic acids and the surface of the nanoparticles are negatively charged, an addition of chaotropic salt, such as NaCl, is required for successful isolation of the target macromolecules. Chaotropic salts or specifically the cations present after dissociation of the salt in water, act as cross-linkers between nucleic acid molecules and the surface of the magnetic particles (Figure 3).

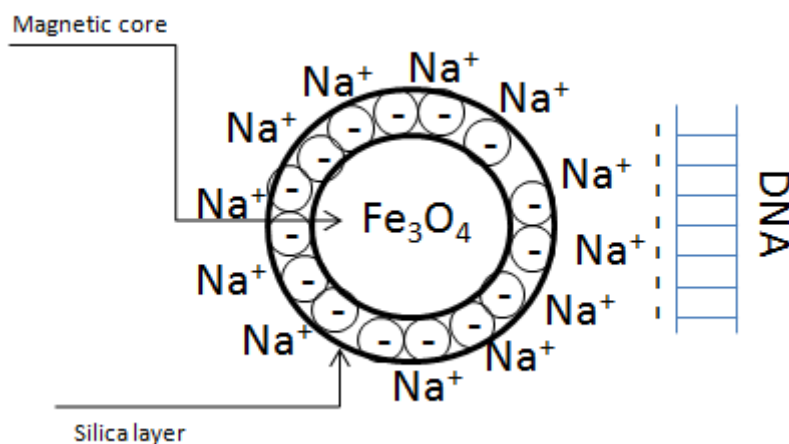


Figure 3: Schematic representation of silica-coated magnetic nanoparticles structure. Negatively charged surface of the magnetic particles interacts with sodium cations present in the binding buffer which acts as a crosslinker for DNA binding.

1.6. Aims of the study

The aim of this project was to evaluate four methods of DNA extraction from human body fluid contaminated soil samples in terms of the quality of DNA profile produced. The four methods were: Phenol/Chloroform extraction, two commercially available kits (PrepFilerTM and PowerSoil®) and magnetic separation using superparamagnetic iron oxide nanoparticles (SPIONs) generated in-house.

2. Materials and methods

2.1. Materials

Materials used in this study included:

2.1.1. Samples

- a) Human blood
- b) Human saliva
- c) Human semen
- d) Soil

2.1.2. Consumables

- a) 1.5 mL Eppendorf tubes
- b) 15 mL Falcon tubes
- c) 0.2 mL PCR tubes
- d) Tips (10, 100, 200 and 1000) μ L and Nishi/Gilson pipettes.
- e) Gloves
- f) Adhesive tape
- g) Universal indicator paper (Sigma, Aldrich, UK)

2.1.3. General reagents

- a) PCR grade water
- b) Distilled, sterile water
- c) Polyvinylpyrrolidone (PVP) (Sigma, Aldrich, UK)

- d) Humic Acid (Sigma, Aldrich, UK)
- e) 4 M Sodium Chloride (Sigma, Aldrich, UK)
- f) 40% Polyethylene glycol (Sigma, Aldrich, UK)
- g) 1 M Tris-HCl buffer(pH=8.0)
- h) 0.5 M EDTA buffer (pH=8.0)
- i) 10% SDS
- j) Proteinase K (QIAGEN, UK)
- k) 1 M DTT
- l) Phenol/Chloroform
- m) NaAc
- n) Ethanol (60%, 70%, 96%)
- o) Isopropanol (100%)
- p) SafeView DNA dye (NBS Biologicals ltd., UK)
- q) Agarose (Sigma, Aldrich, UK)
- r) 1x TAE buffer
- s) 6x Loading dye

2.1.4. PCR reagents

- a) AmpliTaqGold *Taq* (provided with 25 mM MgCl₂ , 10x PCR buffer)
- b) Pfu (recombinant) *Taq* (provided with 50 mM (NH₃)₂SO₄, 10x PCR buffer)
- c) Bio-X-Act Short *Taq* (provided with 25 mM MgCl₂ , 10x PCR buffer)
- d) 4-plex STR kit developed in-house (Nazir et al. unpublished)
- e) Bacterial 16S rDNA primers (F-341 , R-907)
- f) PowerPlex 16 STR kit (Promega, UK)

2.1.5. DNA extraction kits

- a) PowerSoil® MoBio Laboratories Inc., CA, USA)
- b) PrepFiler™ Forensic DNA isolation kit, UK (Applied Biosystems, Warrington, UK)
- c) SPIONs (developed in-house)

2.1.6. Equipment

- a) Laminar flow hood (Bioair Instruments, Italy)
- b) CX-2000 UV crosslinker (Fisher Scientific UK Ltd., UK)
- c) Incubators (HYBAID Ltd, Italy)
- d) Centrifuges (GenFuge, Progen, UK)
- e) Magnetic stand (DYNAL MPC®-E, Oslo, Norway)
- f) Gel electrophoresis tanks (RunOne Electrophoresis cell, CA, USA)
- g) UV-transilluminator (BioDoc-it™-Imaging Systems, CA, USA)
- h) Flat-platform shaker (IKA® Works do Brasil Ltd a , RJ)
- i) NanoDrop 2000 (Thermo Scientific, DE, USA)
- j) GeneAmp PCR system 9700 (Applied Biosystems, UK)
- k) ABI 3500 Genetic Analyzer (Genemapper ID-X software)(Applied Biosystems, UK)
- l) Fridges (Biocold, UK)
- m) Freezers (Biocold, UK)
- n) Vortex (Velp Scientifica, Italy)
- o) qPCR 9700 RT-PCR (Applied Biosystems, UK)

2.2. Methods

In this study, 6x 5mL of semen, 1x 10 mL of blood and 1x 3 mL of saliva were collected from the student. Saliva and semen were collected into several 1.5 mL Eppendorf and 15 mL Falcon tubes, respectively and stored at 4 °C prior to sample preparation. Blood was collected, by a qualified phlebotomist, into a single 10 mL tube containing heparin and stored at 4 °C.

Soil samples (composed of silty-loam soils) were obtained from a local garden (Preston, Lancashire, UK) by Dr Arati Iyengar and kept frozen until the sample preparation step was carried out.

2.2.1. Contamination control

Every precaution was taken throughout the study to minimize the risk of sample contamination.

DNA extraction and PCR sample preparation were always carried out in a pre-PCR laboratory. All body fluids and soil samples were kept in separate sealable plastic bags. In addition, all DNA extractions and PCR amplifications were performed in triplicate alongside negative controls (no body fluid soil control for DNA extractions and no DNA for PCR).

2.2.2. Sample preparation

Prior to the sample preparation, visual and chemical (pH testing) examination of the soil samples was carried out. All samples were prepared by adding 3 mL of liquid saliva, blood or semen to a 15 mL Falcon tube containing 3 mg of soil. The body fluid/soil mixture was then placed for 24 hours at 4 °C to allow complete saturation of the soil. Blanks (negative controls)

were prepared by collecting 50 mg of soil samples prior to addition of the corresponding body fluid.

Body fluid contaminated soil samples of (5, 25, 50, 100, 125 and 250) mg, each in triplicate, were weighed out into sterile, labelled 1.5 mL Eppendorf tubes and subjected to a DNA extraction procedure.

2.2.3. DNA extraction

2.2.3.1. Organic Phenol- Chloroform DNA extraction

Into each tube containing 250 mg of the soil/semen mixture, 460 μ L of the X2 lysis buffer (Appendix 13), 20 μ L of 1 M DTT (Sigma Aldrich) and 20 μ L of Proteinase K was added. Samples were then briefly vortexed and incubated for 1 hour at 56 °C (vortexing every 15 min for 5 sec).

The lysate was collected and subjected to the Organic Phenol: Chloroform DNA extraction procedure (Appendix 6).

2.2.3.2. PrepFiler™ Forensic DNA isolation kit

DNA extraction procedure was carried out on 50 mg of the soil/semen mixture according to the protocol provided with the kit (Appendix 8).

2.2.3.3. PowerSoil® MoBio DNA extraction kit

DNA extraction was performed according to the protocol provided with the kit (Appendix 7), on a range of soil/body fluid samples containing: (5, 25, 50, 100, 125 and 250) mg of the mixture.

A flat-platform shaker was used to perform the bead-beating procedure. Samples were secured and attached to the platform using adhesive tape.

(Note: For samples containing blood and saliva, 250 mg of the body fluid/soil mixture alone was used.)

2.2.3.4. SPION binding based DNA extraction

Into each tube containing (5, 25, 50, 100, 125 and 250) mg of either the soil/semen or soil/blood mixture, 460 μ L of the X2 lysis buffer (Appendix13), 20 μ L of 1 M DTT (Sigma Aldrich) and 20 μ L of Proteinase K was added. Samples were then briefly vortexed and incubated for 1 hour at 56 °C (vortexing every 15 min for 5 sec).

The lysate was collected and subjected to the SPION binding based DNA extraction procedure (Appendix 1). This protocol is henceforth called SPION binding based DNA extraction protocol 1 (Appendix 1).

2.2.3.4.1. Modifications introduced to the SPION binding based extraction protocol

2.2.3.4.1.1. Modification 1 (SPION binding based DNA extraction protocol 2A)

- a) pH and NaCl concentration in the X2 lysis buffer were altered (pH=8.3; 1 M NaCl).
- b) Cold “on ice” precipitation step was added. Following step 3 of the standard SPION binding based DNA extraction protocol, all of the supernatant was transferred to a new Eppendorf tube. The samples were placed on ice, incubated for 10 min and then centrifuged at 13000 rpm for 2 min. The supernatant was transferred to a new tube avoiding the pellet. The next steps were as described in the standard protocol.

Refer to Appendix 1

2.2.3.4.1.2. Modification 2 (SPION binding based DNA extraction protocol 2B)

In addition to modification 1, the following additional modifications were made:

- a) DTT was not used during the lysis step. Step 1 of the standard SPION DNA extraction protocol was altered as follows:

To each tube containing the sample, 480 µL of the X2 lysis buffer and 20 µL of the Proteinase K were added and tube incubated at 56 °C for an hour vortexing every 15 min for 5 sec. Follow the SPION DNA extraction PROTOCOL 2A (Appendix 2).

2.2.3.4.1.3. Modification 3 (SPION binding based DNA extraction protocol 3A)

In addition to modification 1, the following additional modifications were made:

Step 4 of the SPION DNA extraction PROTOCOL 2A was altered. 16% PEG in 4 M NaCl was used as the binding buffer (Appendix 2).

2.2.3.4.1.4. Modification 4 (SPION binding based DNA extraction protocol 3B)

In addition to modification 1, the following additional modifications were made:

- a) Step 5 of the SPION DNA extraction PROTOCOL 2A was altered. 0% PEG in 4 M NaCl was used as the binding buffer (Appendix 2).

2.2.3.4.1.5. Modification 5 (SPION binding based DNA extraction protocol 4A)

In addition to modification 1, the following additional modifications were made:

- a) A pre-lysis washing step was added (20 mM Tris buffer was used) to the SPION DNA extraction PROTOCOL 3A.

1) Step 1. Into each tube containing the soil/semen mixture 500 µL of 20 mM Tris buffer was added and vortexed 5 times for 15 sec at low speed.

Step 2. The samples were then centrifuged at 1000 rpm for 5 min and the supernatant discarded.

Step 3. Steps 1 and 2 were repeated twice followed by the step 1 of the SPION DNA extraction PROTOCOL 3A (Appendix 3).

2.2.3.4.1.6. Modification 6 (SPION binding based DNA extraction protocol 4B)

In addition to modification 1, the following additional modifications were made:

- a) A pre-lysis washing step was added (20 mM Tris buffer was used) (refer to the SPION DNA extraction protocol 4A, Appendix 4).

Note: The pre-lysis washing step was carried out at 35 °C

2.2.3.4.1.7. Modification 7 (SPION inhibitor binding protocol A)

In addition to modification 1, the following additional modifications were made:

- a) A pre-lysis inhibitor binding step was introduced (20 μ L SPIONs and 400 μ L 4 M NaCl was used in this step).

Step 1. Into each tube containing the soil/semen mixture 400 μ L of 4 M NaCl and 20 μ L SPIONs were added. The samples were then placed on a flat-platform shaker and incubated for 10 min at 600 rpm.

Step 2. Using a magnetic stand and blue p1000 tips most of the supernatant and the sample were transferred to a new tube.

Step 3. Steps 1 and 2 were repeated twice followed by the step 1 of the SPION DNA extraction PROTOCOL 3A (Appendix 3).

2.2.3.4.1.8. Modification 8 (SPION inhibitor binding protocol B)

In addition to modification 1, the following additional modifications were made:

- a) Step 8. of the SPION DNA extraction Modification 7 was altered. 10 μ L SPIONs were used during the DNA binding step.

Refer to Appendix 5

2.2.3.4.1.9. Modification 9 (SPION inhibitor binding protocol C)

In addition to modification 1, the following additional modifications were made:

- a) Step 8. of the SPIONS DNA extraction Modification 7 was altered. 5 µL SPIONs were used during the DNA binding step.

Refer to Appendix 5

2.2.3.4.1.10. Modification 10 (SPION binding based DNA extraction protocol 5A)

In addition to modification 1, the following additional modifications were made:

- a) X2 lysis buffer containing 15% PVP was used in the lysis step (Appendix 13).

DNA extraction was carried out according to the SPION DNA extraction PROTOCOL 3A (Appendix 3).

2.2.3.4.1.11. Modification 11 (SPION binding based DNA extraction protocol 5B)

In addition to modification 1, the following additional modifications were made:

- a) X2 lysis buffer containing 15% PVP was used in the lysis step (Appendix 13).
- b) Additional 1.5% PVP was added to the lysate (refer to the SPION DNA extraction PROTOCOL 3A, step 3. DNA extraction was carried out as described in the SPION DNA extraction PROTOCOL 3A (Appendix 3).

2.2.3.4.1.12. Modification 12 (SPION binding based DNA extraction PROTOCOL 5C)

In addition to modification 1, the following additional modifications were made:

- a) X2 lysis buffer containing 10% PVP was used in the lysis step (Appendix 13).

Refer to Appendix 3

2.2.3.4.1.13. Modification 13 (SPION binding based DNA extraction protocol 5D)

In addition to modification 1, the following additional modifications were made:

- a) X2 lysis buffer containing 10% PVP was used in the lysis step (Appendix 13).
- b) Additional 1.5% PVP was added to the lysate (step 3 of the SPION DNA extraction PROTOCOL 3A, Appendix 3).

2.2.3.4.1.14. Modification 14 (SPION binding based DNA extraction protocol 5E)

- a) 1.5% PVP was used in cell extraction procedure. 500 μ L of 1.5% PVP was added to samples containing soil/semen mixture and vortexed for 5 min at low speed.
- b) Samples were then centrifuged at 1000 rpm for 30 sec and the supernatant transferred to a new tube containing 500 μ L of the X2 Lysis buffer (incl. Proteinase K and DTT) following the Modification 3 protocol from step 2.

Refer to Appendix 3

2.2.4. Quantification of DNA

Total DNA in extracted samples was measured using NanoDrop 2000 where 1 μL of each DNA sample was used to carry out the measurements. Prior to each measurement, calibration of the instrument was performed using an appropriate elution buffer.

Amount of human DNA in the samples was measured using the Quantifiler Human DNA quantification kit (Appendix 9) and a real-time 9700 thermo-cycler.

2.2.5. Quantification of humic acid.

Total amount of humic acid content in the extracted DNA samples was measured using Nanodrop 1000 UV-Vis (340 nm).

A standard curve ($R^2 = 0.99$) was created by measuring absorbance (340 nm) of 6 known concentrations (ranging from 10 to 1000 ng/ μL) of commercially bought humic acid (Sigma Aldrich).

2.2.6. Agarose gel electrophoresis

Agarose Gel electrophoresis was carried out by loading 5 μL of DNA extract and 1 μL of 6x loading dye (Thermo Scientific, UK) or 13.5 μL of PCR product and 1.5 μL of the 6x loading dye, onto 1% and 2% agarose gels, respectively, with 5 μL of SafeView dye added. Samples were run for approximately 30 min at 50 V.

Visualisation of the DNA bands on gel was performed using the BioDocit UV-transilluminator.

2.2.7. PCR

PCR amplification was performed as described in 4-plex human, Bacterial 16S rDNA and PowerPlex 16 protocols using a 9700 thermo-cycler.

Refer to Appendix 10: PowerPlex®16 Manual;

Refer to Appendix 11: 4-plex PCR PROTOCOL **for more detail**;

Refer to Appendix 12: Bacterial 16S rDNA PCR PROTOCOL **for more detail**;

2.2.8. Human STR DNA profiling

STR genotyping was performed using the PowerPlex®16 kit and an ABI 3500 Genetic Analyzer Raw data was analyzed using the GeneMapper-IDX Software.

Refer to Appendix 10: PowerPlex® 16 Manual.

Note: Validation of the Powerplex® 16 kit using samples from this study was first performed using full and half volume reactions (Target DNA concentrations were adjusted accordingly). In addition, 0.5 ng, for full reaction and 0.25 ng for half reaction volumes, of the target positive control DNA provided by the PowerPlex® 16 kit was mixed with 10 fold, 20 fold and 30 fold non-target microbial DNA extracted from soil. 1 µL of the mixture was then used to perform PCR (refer to the PowerPlex® 16 Manual).

2.2.9. Statistical analysis

One- , two-way ANOVA and Student T-test analyses were carried out using Excel 2007 to determine significance between and within different sets of data.

3. Results

Four different DNA extraction methods were compared using soil contaminated with three different body fluids (blood, saliva and semen) : phenol/chloroform, SPION binding, PowerSoil® MoBio and PrepFiler™ Forensic DNA isolation kit. The comparison of the DNA purification methods was carried out using a visual examination of the extracted DNA samples on agarose gels as well as PCR-based methods using PowerPlex®16 Human STR kit, 4-plex and bacterial 16S rDNA primers (refer to the Appendix 10, 11 and 12, respectively). Note: 4-plex and bacterial 16S rDNA primers were used for the majority of comparison and optimisation experiments in order to reduce the costs.

3.1. Comparison of DNA extraction methods for isolation of PCR-ready DNA

The results present in Table 4 were obtained from DNA extractions performed on 250 mg samples, containing soil contaminated with semen, using four DNA isolation techniques mentioned above. Results show that the PowerSoil® MoBio DNA extraction kit is most successful in purification of DNA from soil samples. DNA extracted using this method was of high quality (high molecular weight, HMW) and purity (permitting successful PCR amplification of full 4-band human (4-plex) and bacterial (16S rDNA) DNA products without the need of diluting the DNA extracts (Figure 4A).

Extraction of DNA using the Phenol/Chloroform technique resulted in successful isolation of high molecular weight DNA (Figure 4D). However, the DNA extract was dark brown in colour (data not shown) and characteristic dark shadow smears were visible when run on an agarose gel (Figure 4D). In addition, PCR failed to amplify 4-plex and bacterial 16S rDNA

products even when 1 in 10 dilutions of the DNA extracts and increased amount (5U) of *Taq* polymerase were used (note: for increased *Taq* experiments, only one sample out of each triplicate was used to keep the costs down).

PrepFiler™ Forensic DNA isolation kit failed to extract PCR-ready DNA from soil contaminated with semen. Extracted DNA was dark brown in colour (data not shown) and when run on an agarose gel, no DNA bands were present and characteristic dark shadow smears were visible (Figure 4C). PCR amplification targeting 4-plex human and bacterial 16S

Table 4: Results of gel electrophoresis and PCR using DNA extracted using four different methods.

		PowerSoil	Phenol/Chloroform	PrepFiler	SPIONs	
Gel electrophoresis of DNA		Bright HMW DNA, no visible impurities*	Very weak HMW DNA, very high content of impurities*	No visible HMW DNA, very high content of impurities*	3 weak and hardly visible bands- very high content of impurities	
					- DTT (on ice incubation)	+ DTT (on ice incubation)
					No visible HMW DNA, high content of impurities*	Bright HMW DNA, minimal degradation, low content of impurities*
4-plex PCR	Undiluted DNA extract	4/4/4	0/0/0	0/0/0	--	0/0/0
	1:10 DNA dilution	--	0/0/0	0/0/0	--	0/0/0
	Undiluted DNA + 5 U <i>Taq</i>	4	0	0	--	0
	1:10 DNA dilution + 5U <i>Taq</i>	--	0	0	--	4
Bacterial PCR	Undiluted DNA extract	1/1/1	0/0/0	0/0/0	--	0/0/0
	1:10 DNA dilution	--	0/0/0	0/0/0	--	0/0/0

* content of impurities was determined by presence or absence of dark smears on agarose gels.

0/0/0- each digit represent a separate sample of the same triplicate and encodes for the number of bands observed on an agarose gel (a single band for the bacterial 16S rDNA and four bands for the 4-plex amplification reaction). Note: all the amplification reactions carried out with 5U of *Taq* were performed as a single replicate.

rDNA was not successful even with DNA diluted 1 in 10 and addition of 5U of *Taq* polymerase.

DNA extraction performed using the standard SPION binding protocol resulted in DNA extracts which were dark brown in colour (data not shown); and when run on an agarose gel, three bands of very weak intensity were seen and characteristic dark shadow smears were present. “On-ice” precipitation and addition of DTT to the lysis buffer resulted in isolation of DNA of much higher purity and when run on an agarose gel, three bright bands were visible together with some shadow smears being present (Figure 4B). PCR amplification failed to amplify human 4-plex and bacterial 16S DNA products with undiluted as well as 1 in 10 diluted DNA. However, addition of 5U of the *Taq* polymerase as well as 1 in 10 dilution of the DNA extract permitted successful amplification of the 4-plex products.

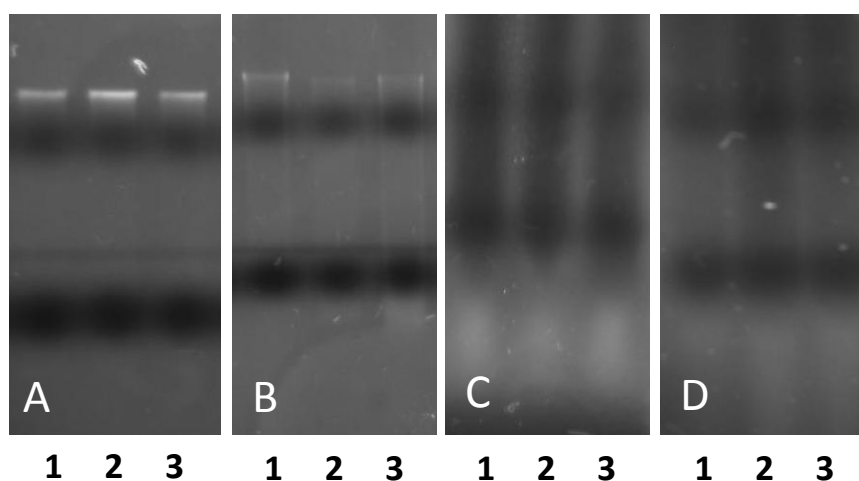


Figure 4: Agarose gel electrophoresis results comparing four different DNA extraction methods. A- DNA extracted using the PowerSoil®MoBio DNA isolation kit; B- DNA extracted using in-house manufactured SPION bindings; C- DNA extracted using PrepFiler™ Forensic DNA isolation kit; D- DNA extracted using organic Phenol/Chloroform technique. 1/2/3- each digit refers to a separate sample of the same triplicate.

DNA extraction using both SPION and the PowerSoil® MoBio DNA isolation kit from soil contaminated with saliva was also performed (data not shown). However, no human DNA was recovered, thus, all comparison studies as well as SPION optimisation experiments were carried out using soil contaminated with semen.

Results show that DNA extracted with the SPION binding protocol 1 (+ DTT) allowed successful amplification of the human 4-plex products. However, the purity of the DNA samples extracted using this method was not sufficient enough to permit PCR on undiluted DNA and standard amount of the *Taq* (0.5 U per reaction). To further investigate this issue, a series of modifications were introduced to the protocol 1 (see following section). In addition, due to the fact that no visible DNA was present and much higher content of impurities was co-extracted (Table 4) when no DTT was used during the lysis step all further DNA extractions involved addition of DTT regardless of the modifications tested.

3.2. Optimisation of the SPION binding based DNA isolation protocol

Several modifications (i.e. indirect lysis, direct lysis, pre-lysis washing, reduction of PEG concentration during SPION binding, pre-lysis SPION based inhibitor binding, and addition of PVP during lysis), of the SPION binding protocol were compared in terms of purity and amount of DNA recovered (both human and total DNA) and whether PCR targeting both human and bacterial DNA was successful.

3.2.1. Comparison of direct and indirect DNA extraction approaches

An indirect cell lysis method was compared to several direct cell lysis methods. The results in Table 5 reveal the indirect cell extraction method to be significantly better in reducing the amount of HA co-extracted during the DNA isolation procedure. The total amount of HA detected in the DNA samples extracted using this method was 8.46 ± 1.33 ng/ μ L. In addition, the eluted DNA was not brown (data not shown) and no shadow smears were present when DNA was loaded onto an agarose gel.

In contrast, every direct DNA extraction method tested, gave significantly higher amounts of HA in the DNA samples. Highest yield of HA, 145.64 ± 1.60 ng/ μ L, was detected in DNA samples isolated using SPIONs (pre-lysis washing with 20 mM Tris at 35 °C) followed by 8% PEG (96.41 ± 44.92 ng/ μ L), and 0% PEG (82.30 ± 8.10 ng/ μ L) concentration during SPION based DNA binding, (20 mM Tris pre-lysis washing (66.15 ± 4.28 ng/ μ L) and PVP direct lysis protocols where the total amount of HA ranged from 12.05 ± 1.18 to 19.23 ± 3.85 ng/ μ L.

In addition, during the DNA elution step, change in colour of the elution buffer was observed (dark brown) for all samples with the exception of DNA isolated using the PVP direct lysis procedure (data not shown).

Both direct and indirect lysis methods were also compared in terms of their effectiveness in recovering DNA.

DNA extraction procedures using the direct lysis approaches were most successful in recovering high amounts of DNA. Total DNA yield ranged from 7.83 ± 1.21 ng/ μ L to 92.00 ± 6.42 ng/ μ L for 15% PVP and 0% PEG concentration during SPION based DNA binding, respectively and were significantly higher than those detected in DNA samples extracted using indirect lysis (2.7 ± 1.01 ng/ μ L).

3.2.2. Effects of PEG concentration on DNA yield and purity

DNA extraction using the unmodified SPION binding based DNA extraction protocol (Appendix 1) where 20% final concentration of PEG in the binding buffer was used, was very problematic. Upon addition of the binding buffer to the lysate, the solution became very dense impairing separation of the nanoparticles from the liquid phase. Thus, different concentrations of PEG in the binding buffer were tested. Two binding buffers containing 16% and 0% PEG final concentration were prepared and DNA extraction was carried out according to the SPIONs based DNA isolation Modification 3 (Table 5).

The binding buffer containing 20% PEG contained the highest amount of HA where over 500 ng/ μ L was detected in the DNA samples (data not shown).

DNA samples extracted using 0% PEG in the binding buffer gave the highest DNA yield of 92.00 ± 6.42 ng/ μ L followed by 20% PEG (51.47 ± 2.57 ng/ μ L) and 16% PEG (23.57 ± 1.86 ng/ μ L).

However, when run on an agarose gel, characteristic dark smears were present and PCR failed when undiluted DNA and 5U of *Taq* was used (Table 5). No significant difference in HA concentration was observed between 0% and 16% PEG concentrations ($p > 0.05$, student T-

test). Reduction of PEG from 20% to 16% decreased the amount of HA almost 5 fold, thus 16% PEG in the binding buffer was chosen for subsequent SPION tests .

3.2.3. Approaches to remove inhibitors

3.2.3.1. Addition of polyvinylpyrrolidone (PVP)

Although direct lysis approaches resulted in much higher DNA yields than those employing indirect lysis procedures, the amount of HA co-extracted using these methods was too great to allow any PCR amplification to occur. Thus, four modifications to the SPION based DNA binding Protocol 3A (Appendix 3) were introduced involving addition of 15%/10% PVP to the lysis buffer and additional 1.5%/0% PVP added to the lysate post-lysis (Table 5).

In comparison to other direct lysis approaches, presence of PVP during the lysis step and further addition post-lysis greatly reduced the amount of HA co-extracted from 537.17 ng/ μ L (standard SPIONs binding protocol, data not shown) to 12.05 \pm 1.18 ng/ μ L , 15.39 \pm 1.54 ng/ μ L, 18.94 \pm 3.11 ng/ μ L and 19.23 \pm 3.85 ng/ μ L for samples containing 15% PVP (during cell lysis) and 1.5% PVP (post-lysis), 10% PVP (during cell lysis), 15% PVP (during cell lysis) and 10% PVP (during cell lysis) and additional 1.5% PVP (post-lysis), respectively. However, total DNA amount recovered (11.9 \pm 1.49 ng/ μ L) was at least two times lower than those with direct lysis methods containing no PVP (23.57 \pm 1.86 ng/ μ L). Furthermore, no visible DNA was present when run on an agarose gel.

3.2.3.2. Pre-lysis washing

The results in Table 5 show that pre-lysis washing of soil samples using 20 mM Tris buffer significantly reduced the amount of HA co-extracted during the isolation procedure compared to the standard SPIONs binding protocol (data not shown).

The total amounts of HA detected were 66.15 ± 4.28 ng/ μ L and 145.64 ± 1.60 ng/ μ L for samples extracted with modifications 5 and 6 of the SPION protocol, respectively. In addition, unlike the DNA extraction using lysis buffers containing PVP, pre-lysis washing steps recovered relatively high amounts of DNA ranging from 71.83 ± 3.04 ng/ μ L to 58.13 ± 2.51 ng/ μ L for samples extracted using modification 6 and 5 of the SPION protocol, respectively.

3.2.3.3. Pre-lysis SPION binding of inhibitors

In this experiment, SPIONs were used before cell lysis to bind HAs. This protocol greatly reduced the amount of humic substance co-extracted during the DNA isolation procedure (Table 5). The total amount of HA detected in the DNA samples was 13.59 ± 2.35 ng/ μ L being almost 40 times lower than those detected in the DNA extracted with standard SPIONs binding Protocol 1. In addition, the total DNA yield in these samples was 9.6 ± 1.54 ng/ μ L, and lower than those extracted using other SPIONs modifications. However, low HA concentration together with sufficient amount of DNA present in these samples permitted successful amplification of the full 4-band product when 1 in 10 DNA dilution was used.

3.2.4. PCR success

The DNA extraction methods were also tested for their ability to recover PCR-ready human DNA using the Quantifiler kit (Tables 5, 7).

Among all the SPION based DNA binding protocols and the commercial kits tested, the direct lysis with pre-lysis washing steps together with PrepFiler™ Forensic DNA isolation kit gave the highest amounts of human DNA ranging from 3.88 ± 1.17 ng/ μ L, 3.39 ± 1.58 ng/ μ L to 7.39 ± 1.94 ng/ μ L for PrepFiler™ (data not shown). The lowest amounts of target DNA were detected in samples extracted using indirect lysis + 1.5% PVP (0.40 ± 0.12 ng/ μ L), direct lysis + PVP (1.31 ± 0.63 ng/ μ L) and the commercial PowerSoil® kit (0.82 ± 0.28 ng/ μ L) (Table 7). However, The PowerSoil® kit was the only DNA isolation method allowing successful amplification of the full 4-plex products from a range of sample amounts (from 25 mg up to the recommended 250 mg of the soil/semen mixture) (Table 7) without the need of performing DNA dilutions or increasing the amount of *Taq* used in each reaction ($C_t < 28$).

The PrepFiler™ Forensic DNA isolation kit failed to extract DNA of sufficient purity to permit PCR amplification even when 1 in 10 DNA dilution and 5U of the *Taq* enzyme was used ($C_t = 39.87$, data not shown).

Among the modifications introduced to the SPION based binding protocol, only the pre-lysis SPION inhibitor binding protocol (Table 5) extracted DNA with sufficient purity to allow PCR amplification with DNA diluted 1 in 10 with no increase in the amount of the *Taq* enzyme ($C_t = 29.71$). However, for the majority of the modifications tested with the exception of the direct PVP lysis method (Modifications 10-13), successful PCR amplification of the 4-plex product occurred when DNA samples were diluted 10 times and 5U of the *Taq* polymerase was used in each reaction.

Furthermore, to eliminate the possibility of PCR failure being due to insufficient concentration of the target DNA rather than inhibition, primers targeting bacterial 16S rDNA were used.

Table 5 shows that no bacterial product was present, across all the modifications introduced to the standard SPIONs protocol, when undiluted DNA was used. However for DNA extracted using modifications 7,10-12 and 14, 1 in 10 dilution of DNA was sufficient to overcome the inhibition.

Table 5: Modifications introduced to the 20 μ L SPION based DNA extractions using 250 mg soil contaminated with semen.

		Pre-lysis washing/SPION based inhibitor binding				Reduction of PEG concentration during SPION based DNA binding		Addition of PVP			
Gel electrophoresis		20mM Tris (room temp)	20mM Tris (35 °C)	SPION binding	PVP (1.5%)	8% final	0% final	Direct lysis (15% PVP)		Direct lysis (10% PVP)	
		Direct lysis (with soil present)			Indirect lysis (no soil present)			1.5% extra to post-lysis supernatant	0% extra to post-lysis supernatant	1.5% extra to post-lysis supernatant	0% extra to post-lysis supernatant
		HMW DNA, less visible impurities	HMW DNA, visible impurities	HMW DNA, no visible impurities	Weak HMW DNA, no visible impurities	HMW DNA, less visible impurities	Weak HMW DNA, visible impurities	No visible bands	No visible bands	No visible bands	No visible bands
DNA[ng/ μ L]		58.13 \pm 2.51	71.83 \pm 3.04	9.6 \pm 1.54	2.7 \pm 1.01	23.57 \pm 1.86	92.00 \pm 6.42	7.97 \pm 0.35	7.83 \pm 1.21	11.9 \pm 1.49	9.57 \pm 0.61
Human DNA[ng/ μ L] (C _i)		3.39 \pm 1.58 (37.41)	7.36 \pm 1.94 (39.04)	1.75 \pm 0.18 (29.71)	0.40 \pm 0.12 (27.85)	2.64 \pm 0.15 (35.04)	2.21 \pm 0.93 (37.01)	0.21 ¹ (31.97)	undetected	1.31 \pm 0.63 (34.92)	0.64 \pm 0.19 (30.78)
HA[ng/ μ L]		66.15 \pm 4.28	145.64 \pm 1.6 ₀	13.59 \pm 2.35	8.46 \pm 1.33	96.41 \pm 44.92	82.30 \pm 8.10	12.05 \pm 1.18	18.94 \pm 3.11	19.23 \pm 3.85	15.39 \pm 1.54
4-plex PCR	Undiluted DNA extract	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
	1:10 DNA dilution	0/0/0	0/0/0	4/4/4	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
	Undiluted DNA + BSA (20 μ g)	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
	Undiluted DNA + 5U <i>Taq</i>	0	0	4	4	3	0	0	0	0	0
	1:10 DNA dilution + 5U <i>Taq</i>	4	4	4	4	3	3	0	0	0	4
Bacterial PCR	Undiluted DNA extract	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
	1:10 DNA dilution	0/0/0	0/0/0	1/1/1	1/1/1	0/0/0	0/0/0	1/1/1	1/1/1	0/0/0	1/1/1

0/0/0- each digit represent a separate sample of the same triplicate and encodes for the number of bands observed on an agarose gel (a single band for the bacterial 16S rDNA and four bands for the 4-plex amplification reaction). Note: all the amplification reactions carried out with 5U of *Taq* were performed as a single replicate.

3.3. Optimisation of the pre-lysis SPION based inhibitor binding protocol

According to the results presented in Table 5, SPION inhibitor binding protocol (Modification 7) was most successful at isolating DNA of sufficient purity to allow 4-plex amplification after 1:10 dilution without the addition of extra *Taq* without compromising the amount of the target DNA recovered.

However, due to the fact that the extraction procedure was highly problematic, where the separation of the magnetic beads from soil particles was very challenging, only approximately half of the sample was successfully transferred to the final step of the lysis procedure (Appendix 5).

Consequently, several sample amounts were tested to assess the optimal sample amount. In addition, different SPIONs amounts (20, 10 and 5 μL) were also tested for the DNA binding step to determine the most suitable amounts of SPIONs for isolation of PCR-ready DNA.

Table 6: Modifications introduced to the SPION based inhibitor binding method for DNA extraction.

	Sample amounts[mg]																	
	5			25			50			100			125			250		
	Amounts of SPIONs used for DNA binding[μL]																	
	20	10	5	20	10	5	20	10	5	20	10	5	20	10	5	20	10	5
Gel electrophoresis	No visible bands			No visible bands			No visible bands			No visible bands			3 very weak bands		No visible bands	3 very weak bands		No visible bands
DNA[ng/μL]	6.87 ±0.71	2.17 ±0.31	1.03 ±0.84	6.10 ±0.26	4.97 ±0.15	4.93 ±0.38	4.27 ±0.47	5.27 ±0.38	3.53 ±0.40	7.67 ±0.31	5.93 ±0.85	6.03 ±0.25	8.13 ±1.05	4.17 ±1.40	5.87 ±1.17	9.13 ±0.67	7.00 ±0.20	5.87 ±0.21
Human DNA [ng/μL] (C _t)	undetected	undetected	undetected	0.21 [†] (27.5)	0.16 [†] (27.63)	0.15 ±0.06 (29.43)	0.78 ±0.40 (28.78)	0.24 ±0.17 (29.98)	0.45 ±0.22 (29.54)	1.43 ±0.70 (29.01)	1.88 ±0.20 (28.63)	0.73 ±0.42 (28.05)	2.01 ±0.27 (31.3)	1.82 ±0.57 (28.09)	1.24 ±0.35 (30.04)	2.27 ±0.19 (30.89)	1.71 ±0.18 (29.04)	1.87 ±1.06 (29.68)
HA[ng/μL]	22.31 ±7.34	15.90 ±2.35	8.72 ±1.18	13.08 ±3.35	10.26 ±1.18	27.95 ±16.45	16.41 ±1.18	15.90 ±4.24	15.90 ±2.35	20.26 ±1.18	24.81 ±1.94	25.38 ±18.67	21.28 ±1.18	12.56 ±0.44	20.77 ±8.03	21.79 ±2.22	14.87 ±0.89	16.41 ±1.94
4-plex	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	4/4/4	0/0/0	0/0/0	0/0/0	0/0/0
4-plex 1:10 DNA dilution	0/0/0	0/0/0	0/0/0	4/4/4	0/3/3	0/4/2	4/4/4	4/0/4	4/1/3	4/4/4	3/0/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4
4-plex 1:10 DNA dilution + 5U <i>Taq</i>	4	0	0	*	0	*	*	0	0	4	4	4	4	4	4	4	4	4
Bacterial 16S rDNA	0/0/0	1/1/0	1/1/1	0/0/0	0/0/0	1/1/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
Bacterial 16S rDNA 1:10 DNA dilution	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1

*- DNA smear on gel (difficult to distinguish the 4 bands)

!- concentrations of human DNA could be determined for two or less samples of triplicate (SE could not be calculated).

0/0/0- each digit represent a separate sample of the same triplicate and encodes for the number of bands observed on an agarose gel (a single band for the bacterial 16S rDNA and four bands for the 4-plex amplification reaction). Note: all the amplification reactions carried out with 5U of *Taq* were performed as a single replicate.

According to the results obtained from DNA extractions from different sample amounts as well as different amounts of SPIONs used for DNA binding step, no significant changes in concentrations of HA were observed. However, no visible DNA was present on an agarose gel when extraction was performed on samples lower than 125 mg and using less than 10 μ L of SPIONs. Highest amounts of total and human DNA were detected in samples extracted from (100, 125 and 250) mg of the soil/semen mixture, ranging from 5.87 ± 0.21 ng/ μ L to 9.13 ± 0.67 ng/ μ L of the total DNA (Table 6). The ratio of total to human DNA did not exceed 10 for all the samples extracted from (100, 125 and 250) mg of the soil/semen mixture regardless of the amount of SPIONs used for the DNA binding step, showing that the SPION extraction method is efficient in isolation of target DNA from nucleic acid mixtures.

Overall, the lower the amount of soil/semen mixture and/or SPIONs used for the DNA extraction the higher the total : human DNA ratio ($p < 0.05$, two-way ANOVA). In addition, no human DNA was detected in the samples extracted from 5 mg of the soil/semen mixture.

This protocol was also tested for its ability to extract PCR-ready DNA. The results in table 6 show that DNA extracted using this method isolate DNA with sufficient purity to permit PCR amplification when 1 in 10 DNA dilution was used.

Although, no amplification occurred when DNA extracted from 5 mg and some samples from 25 mg and 50 mg of the soil/semen mixture was added to the 4-plex reaction mix, amplification of the bacterial 16S rDNA product was successful across all the sample amounts presented in Table 3 (1 in 10 DNA dilution only). In addition, for DNA isolated from 5 mg samples, no DNA dilution was necessary to permit PCR amplification of the 16S rDNA product when 5 μ L and 10 μ L SPIONs was used for the DNA binding step.

In order to directly compare these results with Powersoil®, the entire sample range was also tested using PowerSoil® (Table 7).

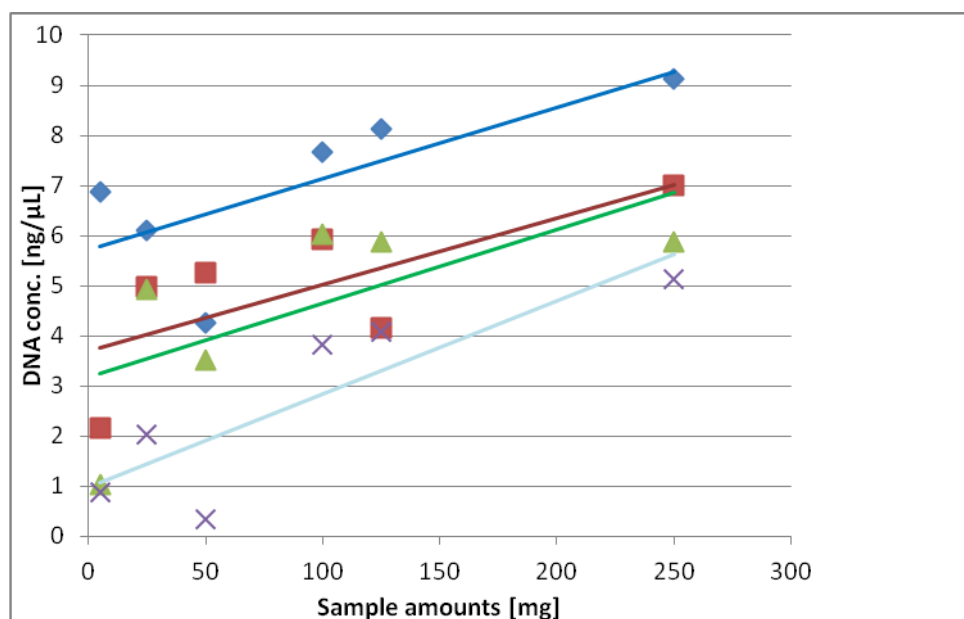


Figure 5: Amounts of total DNA recovered using the commercial PowerSoil® DNA kit and SPIONs, where three different amounts of magnetic beads were used for the DNA binding step.

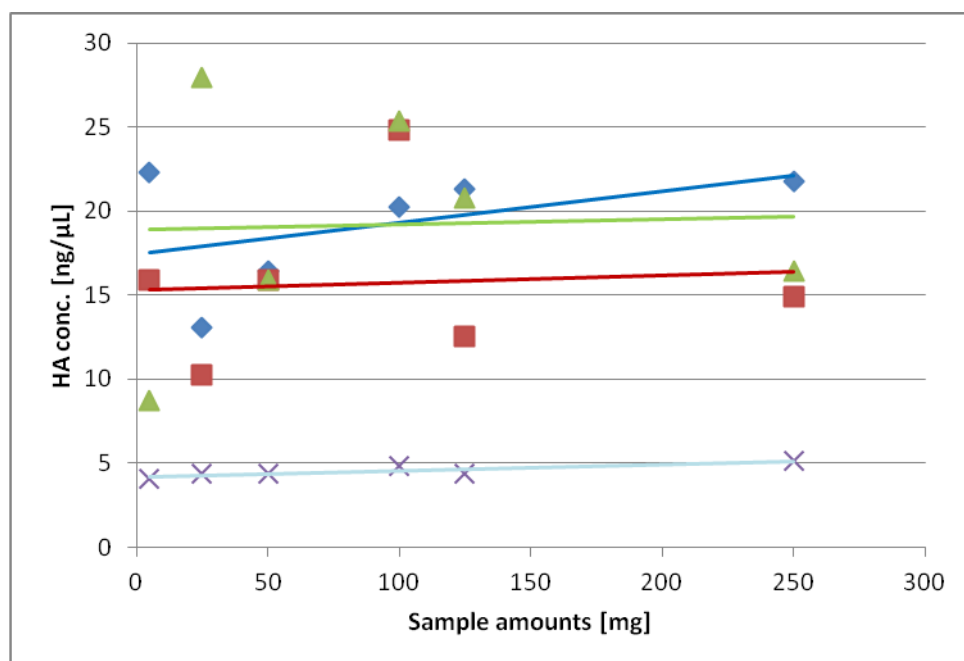


Figure 6: Amounts of HA co-extracted during the DNA isolation procedure using the commercial PowerSoil® DNA kit and SPIONs where three different amounts of magnetic beads were used for the DNA binding step.

- (SPIONs(20ul))
- (SPIONs(10ul))
- (SPIONs(5ul))
- (PowerSoil)

Table 7: DNA extractions on a range of soil/semen sample quantities using a PowerSoil®MoBio kit.

	PowerSoil® MoBio					
	Sample amounts[mg]					
	5	25	50	100	125	250
	No visible DNA	Weak HMW DNA	HMW DNA	HMW DNA	HMW DNA	HMW DNA
Gel electrophoresis						
DNA[ng/μL]	0.87±0.61	2.03±0.68	0.33±0.15	3.83±0.21	4.07±0.25	5.13±0.44
Human DNA [ng/μL] (C₁)	0.03±0.02 (27.67)	0.38±0.07 (27.77)	0.51±0.30 (27.84)	0.19±0.02 (27.76)	0.77±0.15 (27.65)	0.82±0.28 (27.87)
HA[ng/μL]	4.10±0.44	4.36±0.44	4.36±0.44	4.87±0.44	4.36±0.44	5.13±0.44
4-plex	0/0/1	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4
Bacterial 16S rDNA	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1	1/1/1

0/0/0- each digit represent a separate sample of the same triplicate and encodes for the number of bands observed on an agarose gel (a single band for the bacterial 16S rDNA and four bands for the 4-plex amplification reaction). Note: all the amplification reactions carried out with 5U of *Taq* were performed as a single replicate.

Figure 5 shows correlation between sample amounts and the total DNA recovered using the commercial PowerSoil® and SPION based binding methods as well as the amounts of HA co-extracted with these procedures (Figure 6).

As mentioned previously, PowerSoil® DNA isolation kit is most efficient at removing HA from soil extracts. The total amount of HA co-extracted using this kit was far lower than those seen using SPIONs and did not exceed 6 ng/μL regardless of the amount of soil used for DNA extraction (Table 7, Figure 6). However, although the amount of total DNA recovered using PowerSoil® increases with sample amounts, the SPION based DNA extraction technique successfully extracted more DNA across the whole sample range, even when 5 μL of SPIONs was used for the DNA binding step (Figure 5).

3.4. DNA profiling

The Purity of the isolated DNA was also tested by generating a DNA profile using the PowerPlex 16® kit.

Prior to DNA profiling, a validation exercise was performed to assess whether the presence of the large amounts of non-target DNA influenced generation of a good profile using the Powerplex 16® kit and to estimate the variation in TPH and MLB values from different amplifications of the same sample using triplicates.

Target DNA (0.5 ng) was spiked with 5, 10 and 15 ng of microbial DNA extracted from soil using the PowerSoil® DNA extraction kit followed by DNA profiling.

No significant difference in TPH was observed between triplicate samples ($p=0.15$, one-way ANOVA). MLB, significantly decreased with increasing amount of the non-target DNA present ($p=0.02$, one-way ANOVA). However, it appeared like there was no significant difference in MLB variations within each triplicate (data not shown).

Results of DNA profiling of DNA isolated using SPIONs and PowerSoil® are summarised in Figures 11-25 (Appendices 14-28), TPH and MLB values are shown in Figures 7 and 8.

Regardless of the amount of the semen/soil mixture used to perform the DNA extraction, the TPH for PowerSoil®- extracted DNA were at least twice as high as those obtained from corresponding amounts using SPIONs, with a maximum rfu of 28000 (Figure 7).

The highest TPH values for SPION binding based DNA extracts were 11131 and 10784 rfu and were obtained from DNA extracted using 10 µL and 5 µL of SPIONs and 100 mg of the soil/semen mixture respectively.

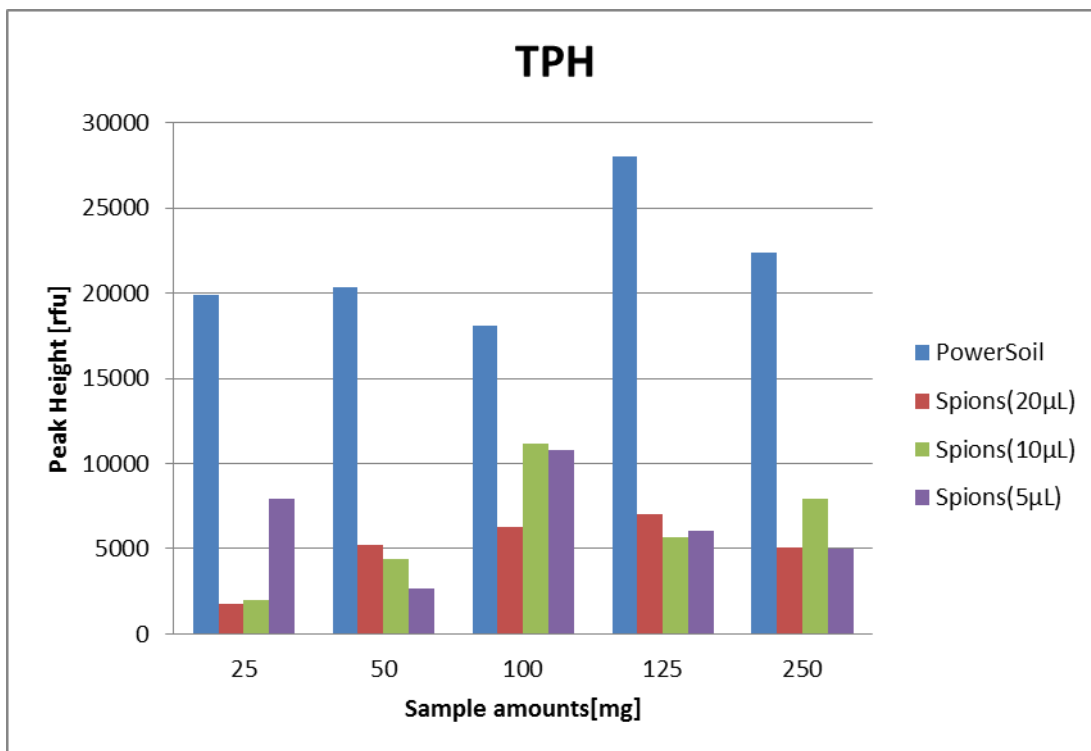


Figure 7: Total peak height (TPH) for DNA profiles generated from DNA extracted using PowerSoil® and SPIONs from 25, 50, 100, 125 and 250 mg of the semen/soil mixture.

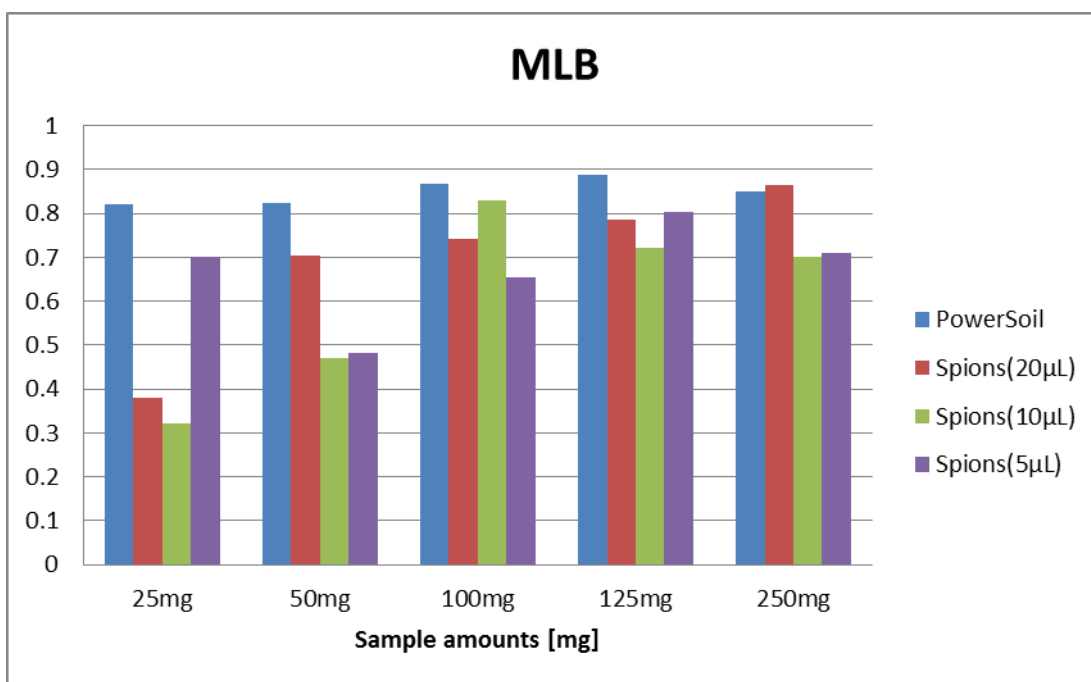


Figure 8: Mean local balance (MLBs) for DNA profiles generated from DNA extracted using PowerSoil® and SPIONs from 25, 50, 100, 125 and 250 mg of the semen/soil mixture.

No clear relationship between the amount of SPIONs used and the TPH values was observed across the whole range of sample amounts. The TPH obtained from 25 mg of the soil/semen mixture and 5 μ L SPIONs is over 3 times as high as those from the other two SPION amounts using 25 mg. The TPH values decrease with decreasing amount of SPIONs in 50 mg samples whereas 10 and 5 μ L SPIONs appear optimal for DNA extraction from 100 mg samples. Figure 8 shows the MLB values for all the DNA profiles generated.

Profiles obtained from DNA extracted using the PowerSoil® kit are highly balanced with MLB values exceeding 0.8 for all sample amounts tested with the highest value of 0.89 observed in 125 mg samples. It appears that the amount of soil/semen mixture used to perform the DNA extraction procedure did not affect the balance of the profiles generated, with the entire range from 5 mg to 250 mg generating highly balanced profiles. Furthermore, the most balanced profile for SPION binding based DNA extraction with a MLB of 0.86, was obtained from 250 mg samples when 20 μ L of SPIONs were used for the DNA binding step; slightly exceeding the MLB values of profiles generated from 250 mg PowerSoil® samples (MLB= 0.84).

MLB values for the profiles obtained from DNA isolated from (100, 125 and 250) mg samples using PowerSoil® are similar to those acquired from SPIONs-extracted DNA.

The SPION protocol where 100 mg sample and 10 μ L of the magnetic beads were used appeared to be the most optimal when both the MLB and TPH are taken into consideration.

Table 8: SPION binding based DNA extractions using a range of blood/soil sample amounts.

	Sample amounts[mg]								
	25			100			250		
	Amounts of SPIONs used for DNA binding[μL]								
	20	10	5	20	10	5	20	10	5
Gel electrophoresis	No visible bands			No visible bands			3 very weak bands	No visible bands	
DNA[ng/μL]	1.27±0.55	0.00	0.00	1.10	0.17±0.06	0.16±0.15	4.60±2.20	0.87±0.21	1.77 ±0.05
Human DNA [ng/μL] (C _I)	0.18±0.02 (29.89)	0.06 (29.35)	0.28 (29.64)	0.09 (30.85)	0.18 (29.82)	0.29 (29.78)	0.38±0.27 (30.97)	0.44±0.22 (29.41)	0.20 (30.01)
HA[ng/μL]	14.36±1.94	8.21±0.44	5.64±0.089	36.15±6.84	8.46±0.77	12.05±0.89	38.97±25.56	6.92±0.77	10.00±1.54
4-plex undiluted DNA	0/0/4	4/4/4	0/0/0	0/0/0	4/4/4	0/4/4	0/4/4	4/4/4	0/0/0
4-plex 1:10 DNA dilution	0/0/0	4/0/0	0/0/0	4/4/4	3/4/4	0/4/4	0/0/0	0/0/0	4/0/0
4-plex undiluted DNA dilution + 5U <i>Taq</i>	0	4	0	4	4	4	4	4	4
Bacterial 16S rDNA	0/0/0	1/1/1	1/1/1	1/0/0	1/1/1	1/1/1	1/1/0	1/1/1	1/1/0
Bacterial 16S rDNA 1:10 DNA dilution	1/1/1	1/0/0	0/0/0	1/1/1	1/0/0	0/0/0	1/1/1	0/0/0	1/1/1

0/0/0- each digit represent a separate sample of the same triplicate and encodes for the number of bands observed on an agarose gel (a single band for the bacterial 16S rDNA and four bands for the 4-plex amplification reaction). Note: all the amplification reactions carried out with 5U of *Taq* were performed as a single replicate.

Table 9: DNA extractions with the PowerSoil® DNA isolation kit (MoBio) using a range of soil/blood sample quantities.

	Sample amounts[mg]		
	25	100	250
Gel electrophoresis	Weak HMW DNA	HMW DNA	HMW DNA
DNA[ng/μL]	1.03±0.40	2.33±0.32	4.10±0.26
Human DNA [ng/μL] (C_t)	0.64±0.13 (27.54)	1.15±0.21 (27.65)	1.45±0.27 (27.64)
HA[ng/μL]	3.85	4.10±0.44	4.87±0.44
4-plex undiluted DNA	4/4/4	4/4/4	4/4/4
Bacterial 16S rDNA	1/1/1	1/1/1	1/1/1

0/0/0- each digit represent a separate sample of the same triplicate and encodes for the number of bands observed on an agarose gel (a single band for the bacterial 16S rDNA and four bands for the 4-plex amplification reaction). Note: all the amplification reactions carried out with 5U of *Taq* were performed as a single replicate.

3.5. Comparison of the optimised SPION based DNA extraction protocol and the PowerSoil® kit for recovery of PCR-ready DNA from blood contaminated soil samples

The previously optimised SPION binding based DNA isolation method and the PowerSoil® kit were also used to compare their ability to recover PCR-ready DNA from different amounts (25, 100 and 250) mg of blood/soil mixtures.

Tables 8 and 9 summarise the results, showing that the Powersoil® kit was much more efficient at both inhibitor removal and DNA isolation. PowerSoil® was able to recover 1.03, 2.33 and 4.10 ng/ μ L total DNA from (25, 100 and 250) mg samples respectively, with HMW bands visible on an agarose gel across the entire sample range (Table 9). In contrast, DNA concentrations isolated using the SPION binding based method ranged from 0.16 ng/ μ L up to 4.60 ng/ μ L (Table 8). No DNA was detected in samples extracted from 25 mg of the blood/soil mixture using 10 and 5 μ L of SPIONs. In addition, no DNA bands were present when run on an agarose gel with the exception of DNA extracted from 250 mg of the soil/blood mixture using 20 μ L SPIONs, where weak HMW DNA bands were visible.

Furthermore, amounts of HA co-extracted during the PowerSoil® DNA isolation procedure did not exceed 5 ng/ μ L (Table 9), whereas over 30 ng/ μ L of the inhibitor was present in samples extracted from 100 and 250 mg using 20 μ L SPIONs or between 5 and 12 ng/ μ L in the remaining sample and SPIONs amounts.

Total amounts of human DNA recovered using SPIONs were relatively low (0.06-0.44 ng/ μ L) as opposed to 1.45, 1.15 and 0.64 ng/ μ L extracted from 250, 100 and 25 mg, respectively, using PowerSoil® isolation kit.

Moreover, unlike previous results using semen/soil samples (Table 6) where no PCR product was detected when undiluted DNA was used, for samples extracted from 250 (20 and 10 μ L

SPIONs), 100 (10 and 5 μ L SPIONs) and 25 mg (10 μ L SPIONs) the 4-band product was visible on an agarose gel. In addition, the bacterial 16S rDNA product was also present for all sample amounts using undiluted DNA with the exception of DNA extracted from 25 mg of the soil/blood mixture using 20 μ L SPIONs where 1 in 10 dilution of DNA was necessary for successful amplification to occur. However, in case of DNA extracted from 250 (10 μ L SPIONs), 100 (10 and 5 μ L SPIONs) and 25 mg (10 μ L SPIONs), DNA dilution appeared to have an adverse effect on PCR success.

In contrast, no DNA dilution was required to permit PCR on DNA extracted using the PowerSoil[®] isolation kit.

3.5.1. DNA profiling

Powerplex[®] 16 profiles generated from DNA isolated using SPIONs and PowerSoil were once again compared. TPH and MLB were evaluated using profiles generated from 250 mg of the blood/soil mixture for both PowerSoil[®] and SPIONs (20 μ L SPIONs used for DNA binding). Most of the profiles produced from DNA extracted using the SPION binding based method, with the exception of 250 mg and 20 μ L SPIONs were unreadable (10 or more loci missing with very low peak heights and high background noise) in contrast to good profiles using Powersoil[®] (data not shown).

TPH (28677 rfu) of triplicate profiles generated from PowerSoil[®]-extracted DNA (250 mg) was over eight times higher than those (3265 rfu) from corresponding sample amounts using SPIONs based DNA binding approach (Figure 9).

Although, the TPH of the profiles acquired from SPIONs-extracted DNA was lower, they were relatively balanced (>0.4) with an MLB of 0.70. However, the purity of DNA extracted using the Powersoil kit was clearly much higher, reflected in both TPH and the MLB (0.94) (Figures 9 and 10).

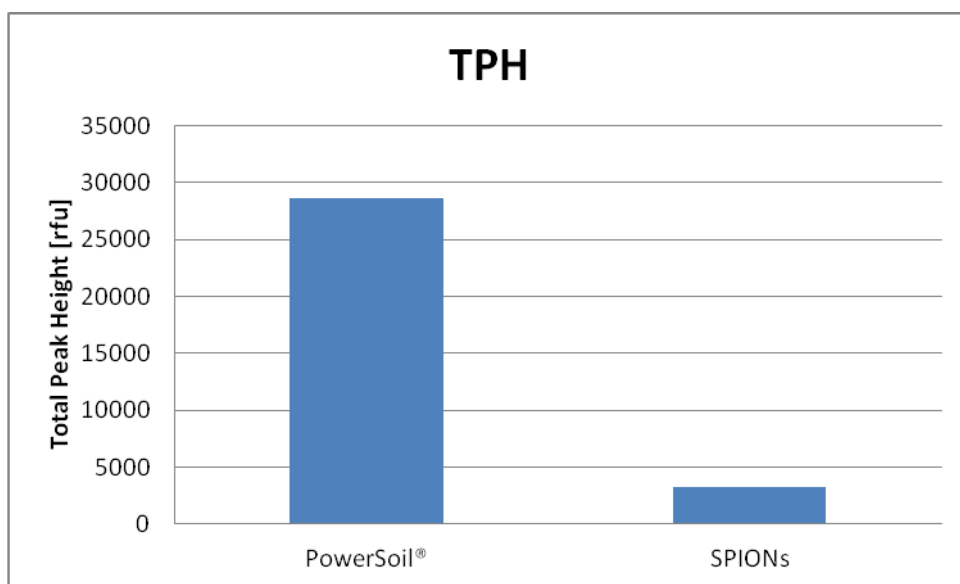


Figure 9: Total peak height (TPH) for DNA profiles generated from DNA extracted using PowerSoil® and SPIONs from 250 mg of the blood/soil mixture.

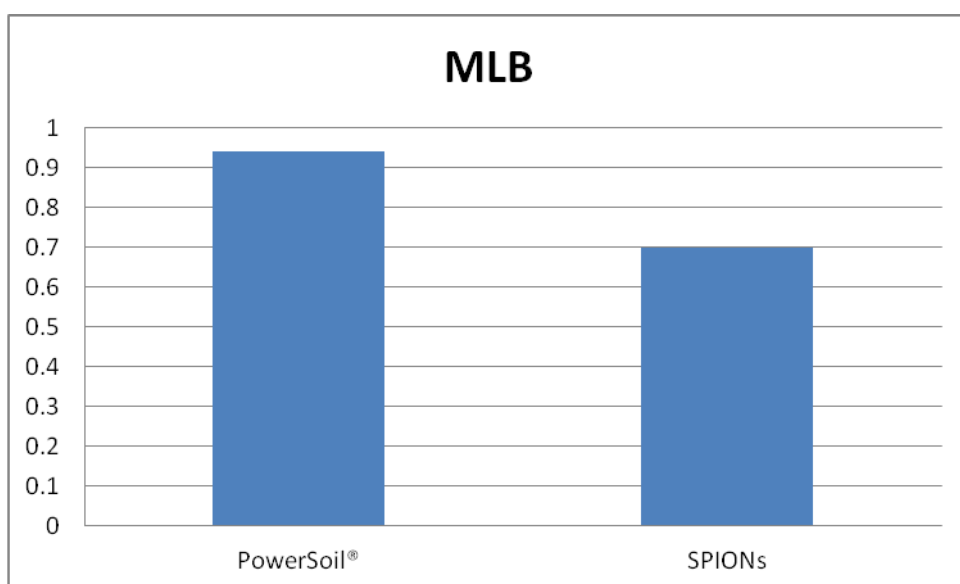


Figure 10: Average mean local balance (MLB) for DNA profiles generated from DNA extracted using PowerSoil® and SPIONs from 250 mg of the blood/soil.

3.6. Assessing PCR inhibition

PCR inhibition was also assessed by using the Quantifiler ® Human DNA quantification kit and looking at the Ct values of the internal positive controls (IPC) for each DNA sample tested.

As the previous results suggested, the DNA samples extracted with the PowerSoil® kit, showed the lowest or none degree of inhibition of the IPC ($C_t < 28$). In contrast, the PrepFiler™ kit extracted DNA with the lowest purity resulting in the highest degree of inhibition among all the DNA extraction method tested ($C_t = 39.87$) (Tables 5-9).

SPION based DNA purification method managed to extract DNA of a moderate purity with the Ct values being in a range between 27.5 and 39.04 depending on the modification used. In addition, among the SPION-based DNA purification methods, the lowest inhibition was detected in the samples extracted with SPION inhibitor binding and the 1.5% PVP indirect lysis protocols ($C_t < 28$) whereas the highest degree of inhibition was observed in the samples extracted with the direct lysis approaches (pre-lysis washing, Table 5).

Furthermore, in comparison to other SPION inhibitor binding DNA purification methods, 125 mg and 10 μ L SPION showed to be most optimal in terms of PCR efficiency. Almost 2 ng of the target DNA together with low amounts of HA (~12 ng/ μ L) ($C_t = 28.09$) allowed successful amplification of the 4-band 4-plex product without the need of dilution (Table 6).

DNA extraction from blood contaminated soil using the SPION inhibitor binding protocol showed slightly higher Ct values comparing to the semen/soil samples (Table 8). However, the amounts of detected HAs were similar (~15-35 ng/ μ L).

4. Discussion

Forensic human DNA profiling has greatly evolved over the last thirty years. The transition from using VNTRs to employing highly sensitive PCR based STR genotyping has been highly beneficial, reducing time and the complexity of analysis as well as the amount of biological material required. However, PCR inhibition remains a major drawback.

Soil is one of the most difficult matrices to extract PCR-ready DNA from, where the significant amount of non-target DNA present is not the only issue encountered. Soils are mixtures of organic and inorganic materials such as minerals, humic substances, a variety of microorganisms, heavy metals or other substances associated with pollution, many of which can potentially inhibit PCR. Thus, effective DNA extraction methods for soil that do not employ time-consuming techniques of sample purification (e.g. size-exclusion chromatography) and result in PCR ready DNA are highly desirable. Moreover, the ideal method would also be suitable for automation, eliminating human error as well as potential cross contamination associated with sample processing.

4.1. Comparison of DNA extraction methods

Four DNA extraction methods were compared, two of which were commercially available DNA extraction kits. The initial comparison of the chosen methods using semen/soil mixtures confirmed previous findings that extraction of PCR-ready DNA from soil samples was particularly difficult due to co-extraction of HAs (Matheson *et al.* 2010, Sebastianelli *et al.* 2008, Spanova *et al.* 2006, Steffan *et al.* 1988). Currently, there are many DNA extraction kits available commercially, however even for some of these (e.g. PrepFiler™ Forensic DNA isolation kit) soil still poses a great challenge in terms of DNA purification.

The initial comparison showed that although phenol/chloroform extraction and the commercial PrepFiler™ were efficient at isolating DNA (over 2 ng of the target DNA was detected by the Quantifiler® Human Quantification kit, data not shown), the sample purification was very poor (Table 1). Unlike PowerSoil®- and SPION based-extraction, sample dilution and addition of 5U *Taq* were not sufficient to overcome the PCR inhibition. The organic phenol/chloroform method relies on organic and water-soluble phase separation and DNA is extracted within the aqueous phase during the phase separation step. The mode of DNA isolation used by the PrepFiler™ kit is most likely based on the charge differences between DNA molecules and the magnetic beads used for the DNA binding step where positively charged PrepFiler™ magnetic beads use the negative charge of DNA molecules for the binding. However, HAs and DNA both share the same chemical features (i.e. charge and solubility) which creates a problem with removal of HAs using these DNA extraction techniques. Both HAs and DNA are water-soluble, negatively charged molecules which co-migrate into the aqueous phase during the organic phenol/chloroform DNA isolation procedure and are both attracted to the positively charged surface of the PrepFiler™ magnetic beads. Despite the fact that both of these methods have been shown to successfully purify HMW DNA from variety of samples such as blood and bones (Brevnov *et al.* 2009), they were unsuccessful at purifying DNA from HA-rich soil in this study. Since the exact composition of the PrepFiler™ magnetic beads is unknown it is difficult to understand the reason behind the failure to extract DNA of sufficient purity. Possibly the increased binding area as well as the affinity of the inhibitor to the surface of the beads caused the experiments to fail at extraction of high purity DNA.

Several researchers have shown that as little as 1 ng of HA can reduce PCR efficiency and 10 ng is sufficient to completely inhibit the reaction (Tien *et al.* 1999, Tsai and Olson 1992b).

Amounts of HA co-extracted using the organic and PrepFiler™ methods were over 50 times higher than the above inhibition threshold (data not shown) as opposed to, on average, only 3 times higher with the SPION binding based approach (Table 6).

The PowerSoil® DNA extraction method was found to be most successful at purification of PCR ready DNA from soil samples regardless of the amount of body fluid/soil used. However, like many another purification methods such as gel filtration or size-exclusion chromatography, DNA yield is usually compromised in favour of high sample purity (Alaeddini 2012, Zhou *et al.* 1996). The amounts of total DNA recovered from the soil/semen mixture using this method did not exceed 10 ng/μL whereas SPION binding based DNA isolation resulted in up to 90 ng/μL of DNA (average 60 ng/μL) (Table 5). In addition, the PowerSoil® purification steps involved several centrifugation and sample re-pipetting steps that are both time consuming and could potentially cause cross contamination. The SPION binding based extraction in contrast, did not require these additional steps.

No positive results were obtained from extraction of DNA from saliva/soil mixture using both PowerSoil® and SPION binding based extraction. On average, amounts of total DNA recovered from 250 μL of saliva ranged between 2.5 and 3.3 ng/μL, which supports previous findings reporting saliva to be a poor source of DNA (Sweet *et al.* 1996, Virkler and Lednev 2009). In addition, a large fraction of the DNA recovered from saliva consists of microbial DNA. Thus, results from this study suggest soil contaminated with saliva to be an unsuitable source of DNA for forensic analysis.

However, since no extensive testing of the soils used in this study was performed (*i.e.* both in terms of their chemical and biological composition) and no comparison to other soils found in the surrounding urban areas were carried out, the results obtained with this study might not

entirely reflect on the usefulness of the DNA purification methods mentioned above (organic DNA purification and PrepFiler™). As mentioned previously (p. 14), various types of soils (sands, silts, clays, loams) contain different amounts of organic matter where the rate of its decomposition can significantly vary. Since HA is produced during decomposition of the dead plant material found in soils, and loamy soils shows the highest rate of decomposition, it could be assumed that the soil samples chosen for this study were particularly rich in HA-like substances resulting in greater co-extraction of HA (as opposed to sands or silts).

4.2. Optimisation of the SPION based DNA binding method

The SPION binding based DNA isolation method performed better than the organic and the commercial PrepFiler™ DNA purification methods with respect to the quantity and purity of DNA extracted. Furthermore, the SPION binding based method was superior to the PowerSoil® kit in terms of DNA yield. In attempts to improve the purity of the DNA extracted using the SPION binding based method, several modifications to the protocol were tested.

Since DNA binding using SPIONs was based on the electrostatic interactions between the bead's surface and both DNA and HA molecules, and no changes to the actual surface of the beads could be introduced, overcoming the issue of co-extraction of HA during the DNA isolation process could only be achieved by : precipitating HAs, modifying the cell lysis step, addition of substances that would enhance binding of DNA and HAs (thus neutralising its negative charge), and altering the amount of sample and/or magnetic beads used for DNA extraction which in turn would enable extraction of sufficient PCR ready DNA suitable for DNA profiling with low concentration of HA permitting successful PCR amplification.

Several modifications were used in conjunction with one another to achieve best results at purifying DNA from HA-rich samples.

4.2.1. Precipitation of HAs

One of the methods of reducing HAs content in extracted DNA is precipitation in the presence of sodium cations at $\text{pH} > 8.0$. Increased pH makes the reaction between HA and sodium ions favourable resulting in production of water insoluble HA salts.

Despite the fact that precipitation of HAs using an on-ice incubation introduced an additional centrifugation step, it reduced the amount of HA co-extracted by more than 20 % (on average 80 ng/ μL) (data not shown) and therefore was kept as an essential step in all further SPION based DNA extractions.

4.2.2. Optimisation of the DNA binding step using PEG

Froehlich and many others found that PEG promotes DNA condensation and can therefore be used as alternative method of DNA isolation when combined with high salt concentration buffers (Froehlich *et al.* 2011). The very feature of PEG causing DNA to aggregate was hoped to facilitate the DNA binding procedure, where the stronger negative charge of the aggregates would have higher affinity to the positively charged surface of the magnetic beads and therefore bind more efficiently reducing the amount of HA being co-extracted. However, a higher viscosity of the solution caused by the binding buffer containing 20% PEG had an adverse effect on DNA purity. Separation of the magnetic beads from the lysate using a magnetic stand separator was impaired by the solution's viscosity in turn making washing steps much more difficult, resulting in sample loss and co-extraction of higher amounts of HA. Reduction of PEG to 16% in the binding buffer decreased the amount of HA in the final DNA extract by over 5 fold but successfully purified over 20 ng/ μL of total DNA which confirmed previous findings by (Spanova *et al.* 2006) that optimal PEG and salt concentrations are crucial in obtaining sufficient amounts of DNA.

Results of HA quantification suggest that total amounts of HA co-extracted were highest when 20% PEG was used in the binding buffer ($> 500 \text{ ng}/\mu\text{L}$) and lowest when either 16% PEG or no PEG was used in the binding buffer (Table 5). Zavarzina *et al.* (2002) found that PEG can adsorb onto silica surfaces through hydrogen-bonding. This could be the cause of larger amounts of HAs being co-extracted in presence of higher concentration of PEG. The amount of total DNA detected when no PEG was used was higher than those extracted with PEG suggesting that the adsorption of PEG onto the SPION surface could have impaired the interaction between the SPIONs and DNA molecules resulting in reduced DNA yield. However, the intensity of the DNA bands on agarose gel was much lower in case of DNA extracted without the presence of PEG in the binding buffer (Table 5). The differences in intensities of the bands could suggest that DNA degradation (shearing) was much more frequent in these samples. Condensation of DNA in the presence of PEG could possibly have a protective effect on DNA molecules preventing shearing during the extraction procedure.

4.2.3. Comparison of direct and indirect lysis

Both direct and indirect lysis methods were compared in terms of DNA yield and HAs content.

Since the indirect lysis method relied on retention of intact cells, which in turn resulted in some sample loss (due to rupture of some cells during pipetting etc.), less target DNA was isolated. The DNA yield was up to 30 fold lower than those extracted using the direct lysis approaches (2.7 ± 1.01 ng/ μ L as opposed to 92.00 ± 6.42 ng/ μ L, Table 2). In addition, HA content in the DNA samples extracted using indirect lysis was on average 10 fold lower than in all direct lysis extractions which support previous findings suggesting indirect lysis to be a more suitable DNA extraction method for recovery of DNA of higher purity from inhibitor rich matrices (Steffan *et al.* 1988). However, many current, commercially available DNA isolation kits are able to isolate DNA of high purity (e.g. PowerSoil®, UltraClean®) without the need of using indirect lysis approaches, and consequently, indirect lysis is no longer in use.

4.2.4. Addition of PVP

PVP has been successfully used in agarose gel-based filtration methods to separate DNA from HAs (Steffan *et al.* 1988, Young *et al.* 1993, Zhou *et al.* 1996). Robe *et al.* has suggested that PVP can retard phenolic compounds that usually co-migrate with DNA molecules during agarose gel electrophoresis. Although, the method was effective in removing HAs from DNA, the steps involved in DNA purification were highly time consuming.

In this study, 15% PVP was added directly into the lysis buffer followed by the standard SPION binding based DNA isolation protocol. The results confirmed previous findings claiming PVP to be an effective method in purification of DNA from HA-rich samples (Young *et al.* 1993)-Addition of PVP (10%, 15%) reduced HA co-extraction by up to 120 ng

per μL of DNA extract in comparison to other direct lysis methods (Table 5). Despite the fact that amounts of HA present in the final extracts was very low ($<20 \text{ ng}/\mu\text{L}$), the method was highly unsuccessful in terms of DNA yield. Presence of 15% and 10% PVP in the lysate seemed to have an adverse effect on binding of both HA and DNA by the magnetic beads and DNA yields were up to 7 times lower than those acquired with other direct lysis methods (Table 2). In addition, much higher sample purity achieved with the indirect lysis method could have also been partially caused by the presence of 1.5% PVP in the buffer, further reducing both the HA and DNA content.

It has been previously suggested that gel filtration using agarose containing PVP caused DNA loss (Young *et al.* 1993). Similar to PEG, PVP could interfere with the DNA binding mechanism by either interacting with the magnetic bead's surface or more likely DNA molecule itself.

4.2.5. Sample pre-treatment

Sample pre-treatment was yet another method tested for reducing HAs. The method was based on the solubility of HAs in aqueous solutions where by performing washes (20 mM Tris buffer) of the soil/body fluid mixture and removal of the liquid phase, a reduction in the amount of HA in the samples could be achieved.

Although, the sample pre-washing step carried out at RT reduced the amount of HA co-extracted by approximately 15% ($66.15 \pm 4.28 \text{ ng}/\mu\text{L}$ compared to over $500 \text{ ng}/\mu\text{L}$ extracted with the standard SPIONs protocol), sample purity was not sufficient to permit successful PCR amplification. In addition, the method involved several centrifugation steps which were both time consuming and could potentially cause cross contamination. In general, sample pre-treatment resulted in the highest DNA yields in comparison to all other modifications tested. A reduction in HA content as a result of the pre-treatment combined with direct lysis

approaches is most likely to be responsible for the increased DNA yield since less HA occupied the SPION surfaces leaving more "binding sites" for DNA molecules.

Moreover, sample pre-treatment carried out at 35 °C resulted in almost 2.5 fold increase in HA content and 1.2 fold increase in DNA yield (Table 5) suggesting that solubility of HAs increases with temperature. This increase in HA was most likely due to the fact that a small fraction of the buffer used to perform the wash step was always left behind after each centrifugation step to ensure that no sample was pipetted out, and as a consequence, larger amounts of HAs dissolved into the buffer and were carried over to the next step of the DNA isolation procedure.

4.2.6. SPIONS based inhibitor binding

Direct lysis methods and sample pre-treatment procedures extracted high amounts of DNA but also HA whereas the indirect lysis method resulted in a much lower amount of DNA but also the smallest fraction of HAs (Table 5). Combining all two methods (i.e. direct lysis and sample-pre-treatment) together with the property of SPIONs to bind both HAs and DNA molecules, a new SPIONs based inhibitor binding protocol was created.

The new extraction method successfully reduced amounts of HA by almost 90% when compared to the standard 16% PEG extraction (8% final PEG) (13.59 ng/μL as opposed to 96.41 ng/μL, Table 5). Although, the total DNA yield suffered 50% decrease compared to the standard 16% PEG extraction, amounts of human DNA were higher than in samples extracted using several other methods (e.g. direct lysis (10% and 15% PVP) and indirect lysis) allowing successful amplification of the 4-plex product with 1 in 10 DNA dilution.

4.2.7. Optimisation of the SPIONs based inhibitor binding protocol- testing sample range and SPIONs amounts

Further optimisation of the SPIONs based inhibitor binding DNA extraction protocol involved reduction of the amount of soil/body fluid sample as well as the amount of SPIONs used for DNA binding. This was carried out in order to find an optimum sample and SPION range for extraction of sufficient amounts of DNA with very low HA content.

As suspected, with increasing amount of the soil/semen mixture and SPIONs, DNA yield increased (Figure 5). However, in terms of HA, the relationship between sample amounts and SPION amounts was less obvious (Figure 6). A certain amount of variability is to be predicted in the binding of HA to SPIONs in the pre-lysis step where the presence of soil may interfere with HA binding.

4.3. PCR success

The DNA extraction methods were also tested for their ability to recover PCR-ready human DNA using the Quantifiler® kit (Tables 5, 7).

The commercial PowerSoil® kit was able to isolate high purity DNA therefore no sample dilution or increase in *Taq* was necessary to permit amplification of both the 4-plex and the bacterial 16S rDNA.

Of all tested methods, the SPION based inhibitor binding method was the only technique that allowed successful amplification of the human and bacterial products when a 10 fold DNA dilution was performed (Tables 5, 6). Further optimisation of the SPIONs based inhibitor binding protocol showed a decrease in DNA yield when less than 100 mg of the soil/semen mixture was used, with 5 mg resulting in undetectable amounts of human DNA and amplification of the 4-plex bands only after addition of extra *Taq* in the case of 20 µL SPIONs (Table 6). The bacterial product in contrast was present across all the sample range

and SPIONs amounts. The optimal amount seemed to be 125 mg of the soil/ semen mixture and 10 μ L SPIONs since that was the only sample that amplified the 4-band product without the need of sample dilution.

The amounts of HAs in the samples extracted using the direct lysis in the presence of 10% and 15% PVP and the indirect lysis were similar to SPION based inhibitor binding method. However, no 4-plex product was detected when PCR was performed on 10 fold dilution of the DNA. The fact that the bacterial 16S rDNA product was successfully amplified using the diluted DNA suggests an insufficient amount of the human DNA template being the cause of PCR failure, rather than PCR inhibition.

Despite the fact that more total DNA was detected in samples extracted without PEG in the binding buffer and no significant increase in HA content was detected, no amplification of the 4-plex product occurred even when 5U of *Taq* was used. In contrast a 3 band product was present when binding of DNA was performed in the presence of 16% PEG in the binding buffer. The presence of a high amount of non-human DNA together with HA was most likely to be the cause of PCR failure in this case. However, the human to microbial DNA ratio was over 5 times lower in these samples compared to 0.11 in DNA extracted in the presence of PEG. Further dilution of the samples extracted without PEG in order to bring the non-target DNA and HA content to a non-inhibitory level, would most likely result in insufficient amount of human DNA. Thus, the binding buffer containing 16% PEG was used in all experiments.

Furthermore, as mentioned previously, soils are very complex, heterogeneous mixtures where their composition can vary between different types of soils (p. 14). Thus, it could be assumed that humic acids were not the only substances affecting the efficiency of PCR amplification. Variety of substances, including different phenolic compounds, heavy metals, inorganic salts,

ions or minerals are known to inhibit *Taq* polymerases. Thus, since the tolerance of both the PowerPlex 16® Human STR kit as well as the 4-plex and bacterial 16S rDNA PCR systems to humic acid was not determined, the conclusions as to what was the main cause of PCR failure were not clear. According to the results of DNA quantification using the Quantifiler kit, there was a slight variation in Ct values between the DNA samples extracted from the blood/soil and semen/soil mixtures despite the fact that the amounts of HAs detected in these samples were fairly similar (Table 6, 8). The observed differences in PCR efficiency were, most likely, caused by the presence of an additional inhibitory substance – heme. Therefore, identification of the exact minimum inhibitory concentrations (MIC) for each system would have explained the reasons behind PCR failure to much higher extent – if the concentration of HA, in certain samples giving negative PCR results, was lower than the MIC for this inhibitor, it could be assumed that a third party inhibitor(s) were also present in the extracted DNA causing PCR failure.

4.4. DNA yield vs HAs co-extraction

Regardless of the DNA extraction method used, a fraction of HAs has always been co-extracted with the DNA. The commercial PowerSoil® kit was found to be most successful at removing HA where on average, the ratio of HA to total DNA was roughly 1:1 across the entire sample range. In addition, despite the fact that PowerSoil extracted less than half the amount of total DNA than that using the SPION binding based isolation method, good quality DNA profiles were successfully generated.

On average, HA content in the samples extracted with direct lysis approaches without the inhibitor binding step, was twice as high as the total DNA amount. DNA and HA could compete for the free binding sites on the surface of the magnetic beads where increased amount of one competitor may result in higher binding efficiency of that particular molecule.

Further modification of the SPION binding based DNA isolation protocol, where an inhibitor binding step was introduced, resulted in reduction of both the total DNA yield and HA content. Additionally, on average the ratio of HA to DNA was 2-4 times higher compared to other SPIONs binding based methods (e.g pre-lysis washing). The results could suggest that during the inhibitor binding step a larger fraction of DNA rather than HA was lost which resulted in higher overall binding efficiency of the inhibitor. This loss of DNA as discussed above, was most likely due to poor retention of intact cells during the pre-lysis procedure.

Similar to the SPION binding based DNA extraction, the commercial PrepFiler™ kit relies on the positive charge of the SPIONs which results in the binding of negatively charged DNA. However, in the case of DNA extraction from soil, PrepFiler™ also binds both the DNA and HA molecules. The initial quantification of HA content in DNA samples extracted using PrepFiler™ and the standard SPION based DNA binding protocol showed no significant difference in concentrations of HA (data not shown) ($p > 0.05$, student T-test). The fact that, in terms of the volume of magnetic beads suspension, more SPIONs were used for DNA binding steps (20 μL and 15 μL for SPIONs and PrepFiler™ respectively) suggests that the affinity of HA to PrepFiler™ magnetic particles was much higher than to SPIONs magnetic beads or that the smaller size of the commercial beads resulted in much higher binding surface area therefore enabling HA to bind more efficiently.

4.5. DNA profiling

4.5.1. Human STR analysis of DNA extracted from soil/semen mixture

DNA profiling performed on the range of sample and SPION amounts showed no significant difference in MLB values for (100, 125 and 250) mg of the semen/soil mixture in comparison to the profiles generated from DNA extracted from corresponding sample amounts using the

PowerSoil® kit (>0.05 , one-way ANOVA). Although, higher HA content in SPION extracted DNA resulted in decreased TPH values in these samples suggesting lower PCR efficiency than that with PowerSoil® ($C_t = 29.68, 27.87$, respectively), the inhibition was not high enough to cause any peak imbalance (<0.4) or allele drop-outs. All peaks met the necessary rfu threshold of 50 for heterozygous and 100 for homozygous loci (refer to PowerPlex® 16 manual). According to the PowerPlex® 16 manual increasing the injection time could result in increased peak heights and therefore peak balance. Consequently this is something that could easily be applied in order to further improve the results using SPIONs. The presence of allele drop-outs is the most crucial characteristic used in judging the quality of DNA profiles generated (Hedman *et al.* 2009) but none were observed in the DNA profiles generated from PowerSoil® and SPIONs inhibitor binding based extracted DNA when (100-250) mg of the mixture and all three SPIONs amounts were used. Highly imbalanced profiles could potentially mislead the analyst of what was a true peak, stutter or an artefact (Hedman *et al.* 2010). Moreover, profile imbalance in conjunction with allele drop-outs could make a particular DNA profile completely unreadable (Hedman *et al.* 2010). Thus, the profiles generated using DNA extracted with PowerSoil® and SPIONs based inhibitor binding protocol in this study were of good quality with none of these issues despite consistently lower overall TPH values with SPIONs.

4.5.2. Human STR analysis of DNA extracted from soil/blood mixture

In the case of blood contaminated soil, when the optimised SPION extraction was used to perform DNA isolation, total DNA yield did not exceed 5 ng/ μ L. In addition, over 50% less target DNA was present in the samples extracted using SPION than those with the PowerSoil® kit (Table 8).

In addition, DNA profiling was successful only when DNA extracted from 250 mg of the blood/soil mixture using 20 μ L SPIONs was used. Although no allele drop-out occurred, the MLB suffered a significant decrease in comparison to the profiles generated using the PowerSoil® kit (Figure 10) (C_t =30.97, 27.64, respectively). In addition, the observed increase in C_t values, compared to DNA extracted from the semen/soil mixture, was most likely due to the presence of heme which together with HAs and reduced DNA yield caused PCR failure and decrease in TPH and MLB. Furthermore, full profiles were obtained from DNA extracted from the entire sample range using PowerSoil®. This difference in performance of the new SPION based extraction method was most likely caused by the fact that semen contains much more DNA. In addition sperm cells are much more resistant to mechanical damage that may be caused by the pre-lysis SPION binding procedure therefore retaining DNA within intact cells rather than loss of DNA from binding to the magnetic beads used for the inhibitor binding step.

5. Conclusions and future work

This study showed that extraction of DNA of good quality and purity from body fluid contaminated soil samples is particularly difficult due to co-extraction of HAs. Even some commercial DNA purification kits PrepFiler™ Forensic DNA isolation kit are incapable of extracting DNA of good quality from body fluid contaminated soil. Others, such as PowerSoil®, are highly effective in removing PCR inhibitors but the amounts of total DNA recovered are low. In addition, the PowerSoil® method often involve several centrifugation and sample re-pipetting steps that are time consuming and could potentially cause contamination.

In this study, an alternative method of extracting DNA using SPIONs has been developed. Although the amounts of HA present in isolated DNA using SPIONs were higher than in PowerSoil®-purified DNA, the method was able to extract at least twice as much DNA for a fraction of the cost (<£1 as opposed to £3.50 per sample). Although overall PCR efficiency was higher when Powersoil®-extracted DNA was used since TPH values were consistently higher, it was also demonstrated that the quality of Powerplex® 16 DNA profiles generated from DNA extracted using both DNA extraction methods were equally good with respects to allele scoring when sample amounts ranged from 100 to 250 mg were compared.

Therefore, the SPION based DNA extraction successfully isolated high amounts of good quality pure DNA from semen contaminated soil samples but additional work on optimisation of the purification protocol is required in order to further reduce the amount of HA co-extracted.

In addition, the SPION inhibitor binding step should ideally be replaced by more efficient DNA purification technique that would allow extraction of nucleic acids from degraded or more sensitive to mechanical damage samples.

Modification of the SPIONs surface to selectively bind target DNA alone (and not HA) would be the most advantageous method of purification reducing PCR inhibition and the sample processing time.

Moreover, much more detailed analysis of the inhibitors present in the extracted DNA samples should also be performed to determine the contribution of each inhibitor to PCR failure which could then be used in further optimisation experiments. It should involve physical and chemical testing of soil samples and DNA extracts to determine their exact composition as well as inhibitor spiking experiments (pure DNA spiked with known concentrations of tested inhibitors) to identify the minimum inhibitory concentration for each inhibitor tested. In addition, different types of soils should also be tested in order to fully understand how their chemical and organic composition affects the purity of extracted DNA.

6. Appendix

Appendix 1

SPIONs binding based standard DNA extraction PROTOCOL 1

1. 460 μ L of the X2 lysis buffer, 20 μ L DTT and 20 μ L Proteinase K were added to Eppendorf tubes containing the sample and vortexed for 5 sec at maximum speed.
2. Samples were incubated at 56 °C for an hour, vortexing every 15 min and then centrifuged at 13000 rpm for 2 min.
3. The supernatant was transferred to a new 1.5 mL tube containing 500 μ L of the binding buffer (20% PEG 4 M NaCl) and 20 μ L of 8.86 mg/mL SPIONs (pre-washed twice in 20mM Tris buffer pH=8.0).
4. DNA binding was carried out for 5 min inverting the samples several times.
5. Magnetic beads were immobilised by placing the samples on a magnetic stand.
6. The supernatant was then discarded.
7. After removing the samples from magnetic stand 300 μ L of 70% ethanol was added and mixed well by inverting 20 times.
8. The samples were then placed back on a magnetic stand to immobilise the magnetic beads and supernatant discarded.
9. Steps 8 and 9 were repeated three times.
10. SPIONs were air-dried at room temperature for no longer than 20 min.
11. DNA elution step was carried out in 100 μ L of 1x TE buffer for 10 min.
12. DNA was stored at -20 °C.

Appendix 2

SPIONs binding based DNA extraction MODIFICATION 1

1. 460 μ L of the X2 lysis buffer, 20 μ L DTT and 20 μ L Proteinase K were added to 1.5 mL Eppendorf tubes containing the sample and vortexed for 5 seconds at maximum speed.
2. Samples were incubated at 56 °C for an hour, vortexing every 15 min and then centrifuged at 13000 rpm for 2 min.
3. Supernatant was transferred to a new 1.5 mL Eppendorf tube and placed on ice for 10 minutes. Samples were then centrifuged at 13000 rpm for 2 min.
4. Supernatant was transferred to a new 1.5 mL tube containing 500 μ L of the binding buffer (20% PEG 4 M NaCl) and 20 μ L of 8.86 mg/mL SPIONs (pre-washed twice in 20mM Tris buffer pH=8.0).
5. DNA binding was carried out for 5 min inverting the samples several times.
6. Magnetic beads were immobilised by placing the samples on a magnetic stand.
7. The supernatant was then discarded.
8. After removing the samples from magnetic stand 300 μ L of 70% ethanol was added and mixed well by inverting 20 times.
9. The samples were then placed back on a magnetic stand to immobilise the magnetic beads and supernatant discarded.
10. Steps 8 and 9 were repeated three times.
11. SPIONs were air-dried at room temperature for no longer than 20 min.
12. DNA elution step was carried out in 100 μ L of 1x TE buffer for 10 min.
13. DNA was stored at -20 °C.

Appendix 3

SPIONs binding based DNA extraction MODIFICATION 3

1. 460 μ L of the X2 lysis buffer, 20 μ L DTT and 20 μ L Proteinase K were added to 1.5 mL Eppendorf tubes containing the sample and vortexed for 5 seconds at maximum speed.
2. Samples were incubated at 56 °C for an hour, vortexing every 15 min and then centrifuged at 13000 rpm for 2 min.
3. Supernatant was transferred to a new 1.5 mL Eppendorf tube and placed on ice for 10 minutes. Samples were then centrifuged at 13000 rpm for 2 min.
4. Supernatant was transferred to a new 1.5 mL tube containing 500 μ L of the binding buffer (16% PEG 4 M NaCl) and 20 μ L of 8.86 mg/mL SPIONs (pre-washed twice in 20mM Tris buffer pH=8.0).
5. DNA binding was carried out for 5 min inverting the samples several times.
6. Magnetic beads were immobilised by placing the samples on a magnetic stand.
7. The supernatant was then discarded.
8. After removing the samples from magnetic stand 300 μ L of 70% ethanol was added and mixed well by inverting 20 times.
9. The samples were then placed back on a magnetic stand to immobilise the magnetic beads and supernatant discarded.
10. Steps 8 and 9 were repeated three times.
11. SPIONs were air-dried at room temperature for no longer than 20 min.
12. DNA elution step was carried out in 100 μ L of 1x TE buffer for 10 min.
13. DNA was stored at -20 °C.

Appendix 4

SPIONs binding based DNA extraction MODIFICATION 5

1. Into each tube containing the soil/seed mixture 500 μ L of 20 mM Tris buffer was added and vortexed 5 times for 15 sec at low speed.
2. The samples were then centrifuged at 1000 rpm for 5 min and the supernatant discarded.
3. Steps 1 and 2 were repeated twice.
4. 460 μ L of the X2 lysis buffer, 20 μ L DTT and 20 μ L Proteinase K were added to 1.5 mL Eppendorf tubes containing the sample and vortexed for 5 sec at maximum speed.
5. Samples were incubated at 56 °C for an hour, vortexing every 15 min and then centrifuged at 13000 rpm for 2 min.
6. Supernatant was transferred to a new 1.5 mL Eppendorf tube and placed on ice for 10 min. Samples were then centrifuged at 13000 rpm for 2 min.
7. Supernatant was transferred to a new 1.5 mL tube containing 500 μ L of the binding buffer (20% PEG 4 M NaCl) and 20 μ L of 8.86 mg/mL SPIONs (pre-washed twice in 20mM Tris buffer pH=8.0).
8. DNA binding was carried out for 5 min inverting the samples several times.
9. Magnetic beads were immobilised by placing the samples on a magnetic stand.
10. The supernatant was then discarded.
11. After removing the samples from magnetic stand 300 μ L of 70% ethanol was added and mixed well by inverting 20 times.
12. The samples were then placed back on a magnetic stand to immobilise the magnetic beads and supernatant discarded.
13. Steps 8 and 9 were repeated three times.
14. SPIONs were air-dried at room temperature for no longer than 20 min.
15. DNA elution step was carried out in 100 μ L of 1x TE buffer for 10 min.
16. DNA was stored at -20 °C.

Appendix 5

SPIONs binding based DNA extraction MODIFICATION 7

1. Into each tube containing the soil/semen mixture 400 μ L of 4 M NaCl and 20 μ L SPIONs were added. The samples were then placed on a flat-platform shaker and incubated for 10 min at 600rpm.
2. Using a magnetic stand and blue p1000 tips most of the supernatant and the sample were transferred to a new tube.
3. Steps 1 and 2 were repeated twice.
4. 460 μ L of the X2 lysis buffer, 20 μ L DTT and 20 μ L Proteinase K were added to 1.5 mL Eppendorf tubes containing the sample and vortexed for 5 sec at maximum speed.
5. Samples were incubated at 56 °C for an hour, vortexing every 15 min and then centrifuged at 13000 rpm for 2 min.
6. Supernatant was transferred to a new 1.5 mL Eppendorf tube and placed on ice for 10 min. Samples were then centrifuged at 13000 rpm for 2 min.
7. Supernatant was transferred to a new 1.5 mL tube containing 500 μ L of the binding buffer (16% PEG 4 M NaCl) and 20 μ L of 8.86 mg/mL SPIONs (pre-washed twice in 20mM Tris buffer pH=8.0).
8. DNA binding was carried out for 5 min inverting the samples several times.
9. Magnetic beads were immobilised by placing the samples on a magnetic stand.
10. The supernatant was then discarded.
11. After removing the samples from magnetic stand 300 μ L of 70% ethanol was added and mixed well by inverting 20 times.
12. The samples were then placed back on a magnetic stand to immobilise the magnetic beads and supernatant discarded.
13. Steps 8 and 9 were repeated three times.
14. SPIONs were air-dried at room temperature for no longer than 20 min.
15. DNA elution step was carried out in 100 μ L of 1x TE buffer for 10 min.
16. DNA was stored at -20 °C.

Appendix 6

Phenol/chloroform DNA isolation PROTOCOL

1. 460 μ L of the X2 lysis buffer, 20 μ L DTT and 20 μ L Proteinase K were added to 1.5 mL Eppendorf tubes containing the sample and vortexed for 5 sec at maximum speed.
2. Samples were incubated at 56 °C for an hour, vortexing every 15 min and then centrifuged at 13000 rpm for 2 min.
3. 250 μ L of the supernatant was transferred into a new tube containing 250 μ L phenol/chloroform and mixed by vortexing for 10 sec at maximum speed.
4. Samples were then centrifuged at 13000 rpm for 2 min.
5. Steps 3 and 4 were repeated twice.
6. The supernatant was transferred into a new tube containing 0.6 volumes of isopropanol and 0.1 volumes of 3 M NaAc, and vortexed for 10 sec at maximum speed.
7. Samples were centrifuged at 13000 rpm for 10 min and supernatant discarded.
8. 250 μ L of 70% ethanol was added to each tube, vortexed and then centrifuged at 13000 rpm for 3 min.
9. Supernatant was discarded and pellets were allowed to air-dry for no longer than 20 min.
10. 100 μ L AE buffer was used to elute the DNA.

Appendix 7

PowerSoil® DNA isolation PROTOCOL (MOBio Laboratories, Inc.)



Experienced User Protocol

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flowthrough and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.
Note: A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x g.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

Appendix 8

PrepFiler™ Forensic DNA isolation kit (Applied Biosystems)

Step 2: Perform lysis

About lysis time and temperature

Incubation times *may* be extended for sample types such as fixed stains that may be difficult to lyse.

In our experience, temperature and time for lysis can be varied between 50 °C and 80 °C and 10 and 90 minutes, respectively. It is recommended that the temperature and time for lysis not exceed 80 °C and 90 minutes, respectively.

Overnight incubation is not recommended due to the potential to degrade DNA.

Perform lysis

After preparing the reagents, perform cell lysis.



WARNING! CHEMICAL HAZARD. PrepFiler™ Lysis Buffer in contact with acids or bleach liberates toxic gases. Harmful if inhaled, absorbed through the skin, and swallowed. Causes eye, skin, and respiratory tract irritation. DO NOT ADD acids or bleach to any liquid wastes containing this product. Avoid breathing vapor. Do not taste or swallow. Use with adequate ventilation. Avoid contact with eyes and skin. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

IMPORTANT! To avoid precipitation of lysis buffer components, do not chill the sample lysate after performing lysis.

1. Bring the thermal shaker temperature to 70 °C.
2. Place a sample in a PrepFiler™ Spin Tube or standard 1.5-mL microcentrifuge tube.
3. To the tube that contains the sample, add:
 - PrepFiler™ Lysis Buffer: 300 µL
 - DTT, 1.0 M: 3 µL (use 5 µL for samples containing semen)

IMPORTANT! If the lysis buffer does not cover the sample substrate (for example, 300 µL may not cover certain types of swabs), bring the lysis buffer and DTT volumes to the volumes specified in [step 4](#) of the large-sample protocol on [page 38](#), then continue following the instructions for the large-sample protocol.

Note: To minimize the number of times you pipette, you can pre-mix the lysis buffer and DTT (1.0 M) for all samples, then add 300 µL of the lysis buffer-DTT mixture to each tube. Prepare a fresh lysis buffer-DTT mixture for each experiment.

4. Cap the tube, vortex it for 5 seconds, then centrifuge it briefly.

5. Place the tube in a thermal shaker, then incubate it at 70 °C and 900 rpm for the appropriate amount of time from the following table:

Lysis times at 70 °C incubation temperature	
Sample Type	Lysis Time (Minutes)
Liquid body fluids	20
Dried stains or samples on swabs	40
Neat semen samples	90

Note: You can use a heat block instead of a thermal shaker. Read [“About thermal shakers” on page 6](#) before using a heat block. If you use a heat block, briefly vortex and centrifuge the tube every 5 minutes.

Step 4: Bind genomic DNA to magnetic particles

After performing cell lysis and, if necessary, removing the sample substrate, add magnetic particles to bind the DNA.



WARNING! CHEMICAL HAZARD. PrepFiler™ Magnetic Particles are harmful by inhalation, skin absorption, and if swallowed. Causes eye, skin, and respiratory tract irritation. Do not taste or swallow. Avoid breathing vapor (or dust). Use with adequate ventilation. Avoid contact with eyes and skin. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

-
1. Allow the sample lysate to come to room temperature (approximately 5 minutes).

IMPORTANT! To avoid precipitation of lysis buffer components, do not chill the sample lysate.

-
2. Vortex the PrepFiler™ Magnetic Particles tube approximately 5 seconds, invert the tube to confirm that no visible pellet remains in the bottom of the tube, then centrifuge briefly.

Note: If you are pipetting multiple samples, vortex the magnetic particles tube every 5 minutes until you complete the next step.

-
3. Pipette 15 µL of magnetic particles into the tube containing the sample lysate.
 4. Cap the sample lysate tube, vortex it at *low* speed (approximately 500 to 1,200 rpm) for 10 seconds, then centrifuge it briefly.

IMPORTANT! This step is required before you add isopropanol in order to promote binding.

-
5. Add 180 µL of isopropanol to the sample lysate tube.
 6. Cap the sample lysate tube, vortex it at *low* speed (approximately 500 to 1,200 rpm) for 5 seconds, then centrifuge it briefly.
 7. Place the sample lysate tube in a shaker or on a vortexer (with adaptor), then mix at room temperature at 1,000 rpm for 10 minutes.

Step 5: Wash bound DNA

After binding the DNA to the magnetic particles, wash the magnetic particles to remove impurities and inhibitors.



WARNING! CHEMICAL HAZARD. PrepFiler™ Wash Buffer

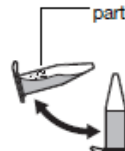
Concentrate causes eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Vortex the sample DNA tube:

- If magnetic particles are present on the sides of the sample DNA tube above the meniscus, invert the tube to resuspend the particles.
- Vortex the sample DNA tube at maximum speed (approximately 10,000 rpm) for 10 seconds, then centrifuge briefly.



If magnetic particles are present above the meniscus, then invert the capped tube to resuspend the magnetic particles.



Note: It is acceptable to have magnetic particle aggregates suspended in the solution or on the side of the tube below the meniscus.

2. Confirm that the magnet in the magnetic stand is properly aligned.

Note: See “Magnetic stand guidelines” on page 13.

3. Place the sample DNA tube in the magnetic stand and observe that the magnetic particles form a pellet against the back of the tube. Wait until the size of the pellet stops increasing (approximately 1 to 2 minutes).



The pellet forms against the magnet at the back of the tube

Note: Samples containing high levels of proteins or other impurities may require more time.

Note: For some sample types such as blood, the solution may remain colored after the magnetic particles are separated.

4. With the sample DNA tube remaining in the magnetic stand, use a pipette to carefully remove and discard *all* visible liquid phase.

IMPORTANT! When removing the liquid phase, do not aspirate magnetic particles or disturb the magnetic particle pellet.

Note: One way to remove the liquid phase is to use a size P200 or P1000 pipettor to remove most of the liquid, then use a size P20 pipettor to remove the remaining liquid.

5. Perform steps a through e *three* times:
 - a. Add 300 μ L of prepared PrepFiler™ Wash Buffer to the sample DNA tube.
 - b. Cap the sample DNA tube and remove the tube from the magnetic stand.
 - c. Vortex the sample DNA tube at maximum speed (approximately 10,000 rpm) until there is no visible magnetic particle pellet on the side of the tube (approximately 5 seconds), then centrifuge briefly.

Note: It is acceptable to have magnetic particle aggregates suspended in the solution.

- d. Place the sample DNA tube in the magnetic stand for 30 to 60 seconds.
 - e. With the sample DNA tube remaining in the magnetic stand, use a pipette to carefully remove and discard *all* visible liquid phase.

IMPORTANT! When removing the liquid phase, do not aspirate magnetic particles or disturb the magnetic particle pellet.

6. With the sample DNA tube remaining in the magnetic stand, open the tube, then allow the magnetic particles-bound DNA to air-dry for 7 to 10 minutes.

IMPORTANT! Air-drying for more than 10 minutes may reduce DNA yield.

IMPORTANT! If the room temperature is $>25^{\circ}\text{C}$, reduce the drying time to 5 minutes.

Step 6: Elute DNA

After performing the wash step, resuspend the purified DNA and separate the DNA eluate from the magnetic particles.

1. Bring the thermal shaker temperature to 70 °C.
2. Add 50 µL of PrepFiler™ Elution Buffer to the sample DNA tube.

Note: Do *not* use water instead of PrepFiler™ Elution Buffer. In place of PrepFiler™ Elution Buffer, you can prepare low TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) or purchase low TE buffer from Teknova (Cat # T0223).

3. Cap the sample DNA tube, vortex it at maximum speed (approximately 10,000 rpm) until there is no visible magnetic particle pellet on the side of the tube (approximately 5 seconds), then centrifuge it briefly.
4. Place the sample DNA tube in a thermal shaker, then incubate at 70 °C and 900 rpm for 5 minutes.

Note: You can use a heat block instead of a thermal shaker. Read [“About thermal shakers”](#) on page 6 before using a heat block. If you use a heat block, briefly vortex and centrifuge the tube every 2 to 3 minutes.

5. Vortex the sample DNA tube at maximum speed (approximately 10,000 rpm) until there is no visible magnetic particle pellet on the side of the tube (approximately 2 seconds), then centrifuge briefly.
6. Place the sample DNA tube in the magnetic stand, then wait until the size of the pellet at the side of the tube stops increasing (at least 1 minute).
7. Pipette the liquid in the sample DNA tube (which contains the isolated genomic DNA) to a new spin tube or 1.5-mL microcentrifuge tube for storage.

IMPORTANT! When removing the liquid phase, do not aspirate magnetic particles or disturb the magnetic particle pellet.

Note: The isolated DNA can be stored at 4 °C for up to one week, or at –20 °C for longer storage.

8. If the eluted DNA extract is turbid (for example, this may occur in tissue samples with a high fat content), centrifuge the tube for 5 to 7 minutes at maximum speed (approximately 10,000 rpm), then transfer the clear supernatant to a new 1.5-mL microcentrifuge tube.

Appendix 9

Quantifiler® Human DNA quantification kit PROTOCOL (Applied Biosystems)

Preparation of DNA standards dilution series:

1. Label eight sterile eppendorf tubes and Std 1 – Std 8.
2. Dispense 30 µl of sdH₂O into Std tube 1 and 20 µl into each of the remaining tubes (Std 2 – Std 8).
3. Preparation of Std 1:
 - a. Thaw the Quantifiler Human DNA Standards and vortex to ensure it is thoroughly mixed.
 - b. Add 10 µl of Quantifiler Human DNA to Std tube 1.
 - c. Mix thoroughly.
4. Preparation of Std 2 – Std 8:
 - a. Remove 10 µl from Std 1 and add to Std tube 2.
 - b. Mix thoroughly.
 - c. Remove 10 µl from Std 2 and add to Std tube 3.
 - d. Mix thoroughly.

Repeat steps 4c and 4d for the remained of the dilution series.

Concentration of DNA Standards:

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
ng/µl	50	16.7	5.56	1.85	0.62	0.21	0.07	0.02

Real-Time PCR Protocol :

Step 1: Preparation of DNA samples.

1. Calculate the required volume for each component (i.e. multiply the volume below by the number of samples).

Component	Volume per Reaction (µl)	Volume for ____ reactions
Quantifiler Human Primer Mix	5.25	
Quantifiler PCR Reaction Mix	6.25	

2. In a sterile eppendorf tube make up a master mix of the above components for all your reactions.
3. Place a 96 well optical plate in its holder – this is important to prevent contamination of the base of the plate which may affect the results.
4. Pipette 11.5 µl of the master mix into each plate well.
5. Using a new sterile tip each time add 1 µl of sample/standard to the aliquoted master mix. NOTE: set standards in duplicate.
6. Ensure there are no air bubbles at the bottom of your tubes and that all liquid is at the bottom of the tube.
7. Place the optical adhesive cover on the 96 well plate. Make sure that the cover is securely adhered to the wells. The plate is ready to be placed in the machine.
8. Load the optical plate into the 7500 (NOTE: well A1 should be in the top left corner).

Step 2: RealTime-7500 Set up

1. Start the 7500 Software System.
2. Select > FILE > NEW

You should see a New Document Wizard:

Assay = Absolute Quantification (Standard Curve).
Container = 96 Well – Clear.

Template = Blank Document.

Give your plate a name.

1. Select > NEXT.
2. Select the appropriate detector(s)

IPC = VIC

Quantifiler Human Kit = FAM

Click > ADD

3. Select > NEXT.
4. Specify the wells and that you are going to use and which detectors are to be used for each well.
 - a. Highlight the wells.
 - b. Click USE for each detector.
 - c. Under the TASK select whether the sample is a Standard or an Unknown.
 - d. If a Standard enter the Quantity as appropriate.
5. Select > FINISH.
6. You should now see a plate setup, there are several tabs:
 - a. Setup Tab – shows you your plate.
 - b. Instrument Tab – Shows the cycling parameters (can be edited if required) – **set the reaction volume**

We will use the default Cycling parameters:

95°C 10 min

95°C 15 sec

60°C 1 min




- c. Results Tab – will detail the results of your analysis.
7. In the Setup Tab
 - a. Double click on a well – this will open the well inspector.
 - b. Enter your sample names into each appropriate well

Detectors, Tasks and Quantities can be edited at this point if required.

8. Select > CLOSE.
3. Select > FILE > SAVE AS and save your file.

4. Select > START

Step 3: Analysis of results:

1. Open 7500 Software System.
2. Select > FILE > OPEN > find your file.
3. You should now see a plate setup with several tabs:
 - a. Setup Tab – shows you your plate.
 - b. Instrument Tab – Shows the cycling parameters.
 - c. Results Tab – will detail the results of your analysis.
4. Select  to analyse your data.
5. Select the Results Tab. Within the results tab are many other tabs:
 - a. Plate – shows your plate set up.
 - b. Spectra – shows the raw data (all wells at once).
 - c. Component – can select data from single wells.
 - d. Amplification plot – can show your data as single wells or selected wells in either log or linear view.
 - e. Standard Curve – shows the results from the standards against which you unknown samples will have been quantified.
 - f. Report - shows the quantification results from your samples.
6. Select FILE > EXPORT allows you to export your results data in Excel format.



Appendix 10

PowerPlex 16® Human STR kit PROTOCOL (Promega)

Step 1- PowerPlex 16 PCR Set up:

	per reaction
Buffer Mix	2.5 µl
Primer Mix	2.5 µl
Amplitaq GOLD	0.8 µl
H ₂ O	19.2 µl
DNA	1.00 µl

PowerPlex 16 PCR Cycling Parameters:

Initial denaturation:	95°C 11 mins		
	96°C 1 min		
Denaturation:	94°C 30 sec 100% ramp		for 10 cycles
Annealing:	60°C 30 sec 29% ramp		
Extension:	70°C 45 sec 23% ramp		
Denaturation:	90°C 30 sec 100% ramp		for 22 cycles
Annealing:	60°C 30 sec 29% ramp		
Extension:	70°C 45 sec 23% ramp		
Final Extension:	60°C 30 mins		
Hold:	4°C		

Step 2 – Sample Preparation

7. For each sample you will require 10 µl Formamide + 0.5 µl ILS600 size standard. Make up a master mix sufficient for all samples
8. Aliquot 10.5 µl of master mix into 0.2 ml eppendorf tubes and add 1 µl of PCR sample or Allelic Ladder
9. Denature, vortex to mix and spin
10. Remove the lid and set tubes in tray with septa and lid

Step 3 – Preparing the PowerPlex16 Run

11. Go to Window>Manual Control>Temperature Set>Set at 60°C then execute
12. Go to File> New, Select Genescan Sample Sheet 96
13. Add you sample names into the appropriate wells
14. Cut and Paste data into the Sample Info column
15. Save As>FILENAME and close
16. Go to File>New>Genescan Injection List and find your sample sheet
17. Make sure settings are correct:
 - Module = GS STR POP4 (A)
 - Injection = 5
 - Run Time = 32 min



Step 4 – Genemapper

19. Open Genemapper
20. Go to File>Add Samples to Project
21. Find data, Computer>D:>AppliedBio>3.0>Runs>find file by DATE
22. Add to list
23. Check that settings are correct
 - Sample type = either 'Sample' or 'Allelic Ladder'
 - Analysis Method = e.g. microsatellite default
 - Panel = chose appropriate multiplex kit if appropriate (Powerplex 16)
 - Size = chose the correct size standard (ILS600)
 - Matrix = as appropriate (Powerplex16)
24. Save As>FILENAME



Appendix 11

4-plex PCR set up PROTOCOL

Background information:

4-plex is an in-house developed PCR multiplex amplifying four different fragments including 70 bp, 194 bp, 305 bp and 384 bp fragments in a single closed-tube reaction format. The amplification protocol have been optimized and successfully used to assess effects of tissue degradation on PCR efficiency in humans, rabbits and pigs (Nazir *et al.* 2013). Note that the 4-plex is not a STR type of multiplexes therefore cannot be used for individualization.

Total volume of the Primer Mix (20 µL) = 7.5 µL of the 4-plex primers mix (A + B + C + D) + 12.5 µL H₂O

Primer set	Amplicon length	Final conc. in the Primer mix	Volume from 100 µL stock
A	70bp	0.1 µM	0.5 µL F 0.5 µL R
B	194bp	0.1 µM	0.5 µL F 0.5 µL R
C	305bp	0.15 µM	0.75 µL F 0.75 µL R
D	384bp	0.4 µM	2 µL F 2 µL R
	TOTAL		7.5 µL

AmpliGOLD MASTER MIX

REAGENT	VOLUME	
10x buffer	1.5 µL	} x NO. reactions
MgCl ₂ (25mM)	1.5 µL	
dNTP(25mM)	0.12 µL	
Primer Mix	0.6 µL	
TaqGold	0.1 µL	
H ₂ O	10.18 µL	
DNA	1 µL	
TOTAL	15 µL	

PCR CYCLIN CONDITIONS

95 °C	5mins	} 30 cycles
94 °C	1min	
60 °C	1min	
72 °C	1min	
72 °C	20mins	

Appendix 12

Bacterial 16S rDNA PCR set up PROTOCOL

Background information

Primers targeting a single 16S rDNA fragment of 566 bp were used in this thesis as a DNA extraction control method to ensure that any given PCR failure was not caused by lack of DNA template.

Primers used:

F-341 5'-CCTACGGGAGGCAGCAG

R-907 5'-CCGTCAATTCMTTGTGAGTTT

AmpliGOLD MASTER MIX

REAGENT	VOLUME	
10x buffer	1.5 µL	} x NO. reactions
MgCl ₂ (25mM)	1.5 µL	
dNTP(25mM)	0.12 µL	
Primer Forward	0.03µL	
Promer Reverse	0.03 µL	
TaqGold	0.1 µL	
H ₂ O	10.18 µL	
DNA	1 µL	
TOTAL	15 µL	

PCR CYCLIN CONDITIONS

95 °C	5mins	} 30 cycles
95 °C	0.5min	
60 °C	0.5min	
72 °C	0.5min	
72 °C	20mins	

Appendix 13

X2 Lysis Buffer

EDTA (ph=8.0)	20 mM
Tris-HCL	20 mM
NaCl	1.0 M
SDS	2%
DTT	80 mM
Proteinase K	400 µg

Note: For 15% and 10% PVP X2 lysis buffers, 15 and 10 g of PVP were added to 100 mL of the X2 lysis buffer, respectively.

Appendix 14

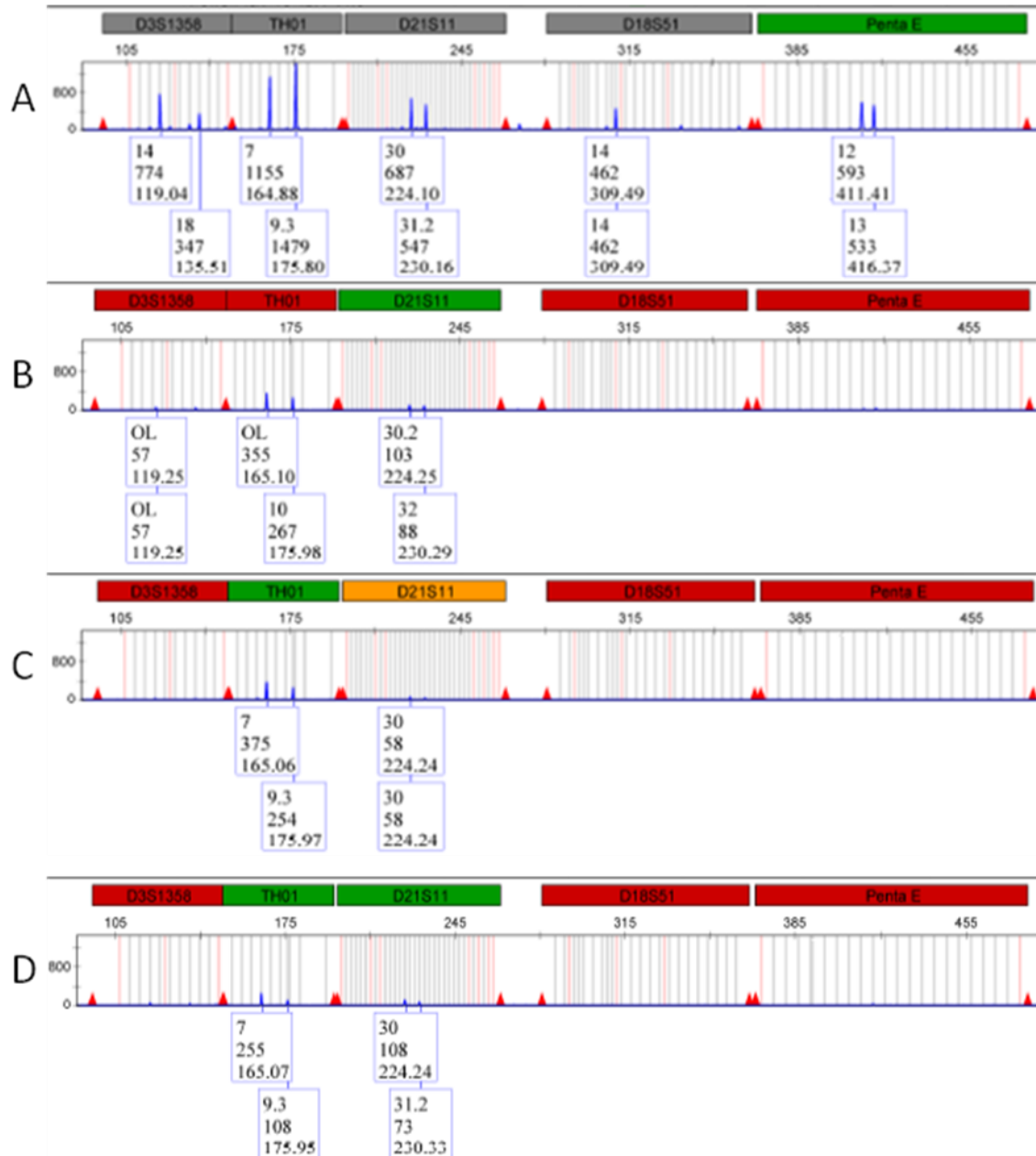


Figure 11. STR profile using DNA extracted from 25mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 15

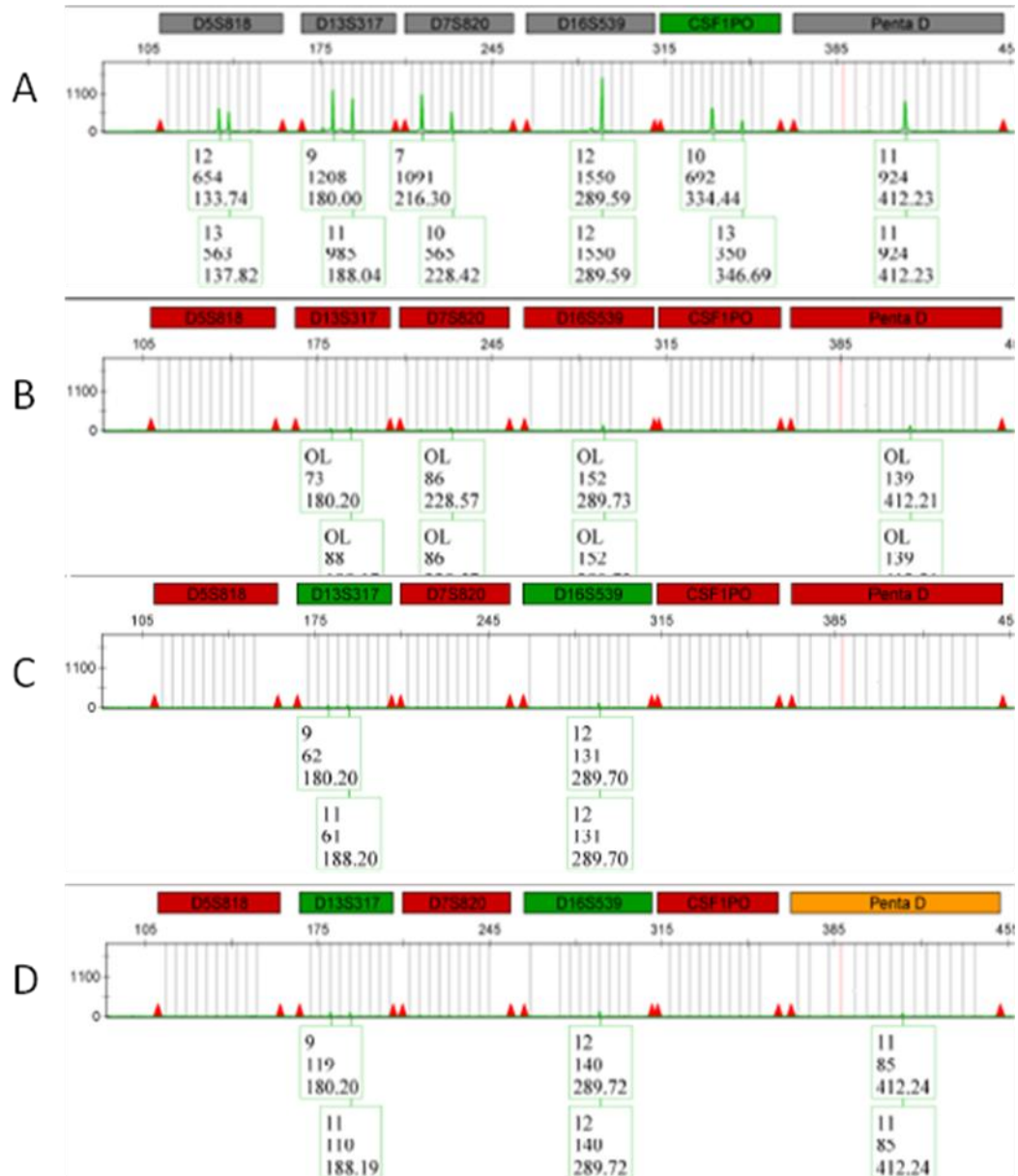


Figure 12. STR profile using DNA extracted from 25mg of the soil/seed mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 6 of the 16 loci are shown.

Appendix 16

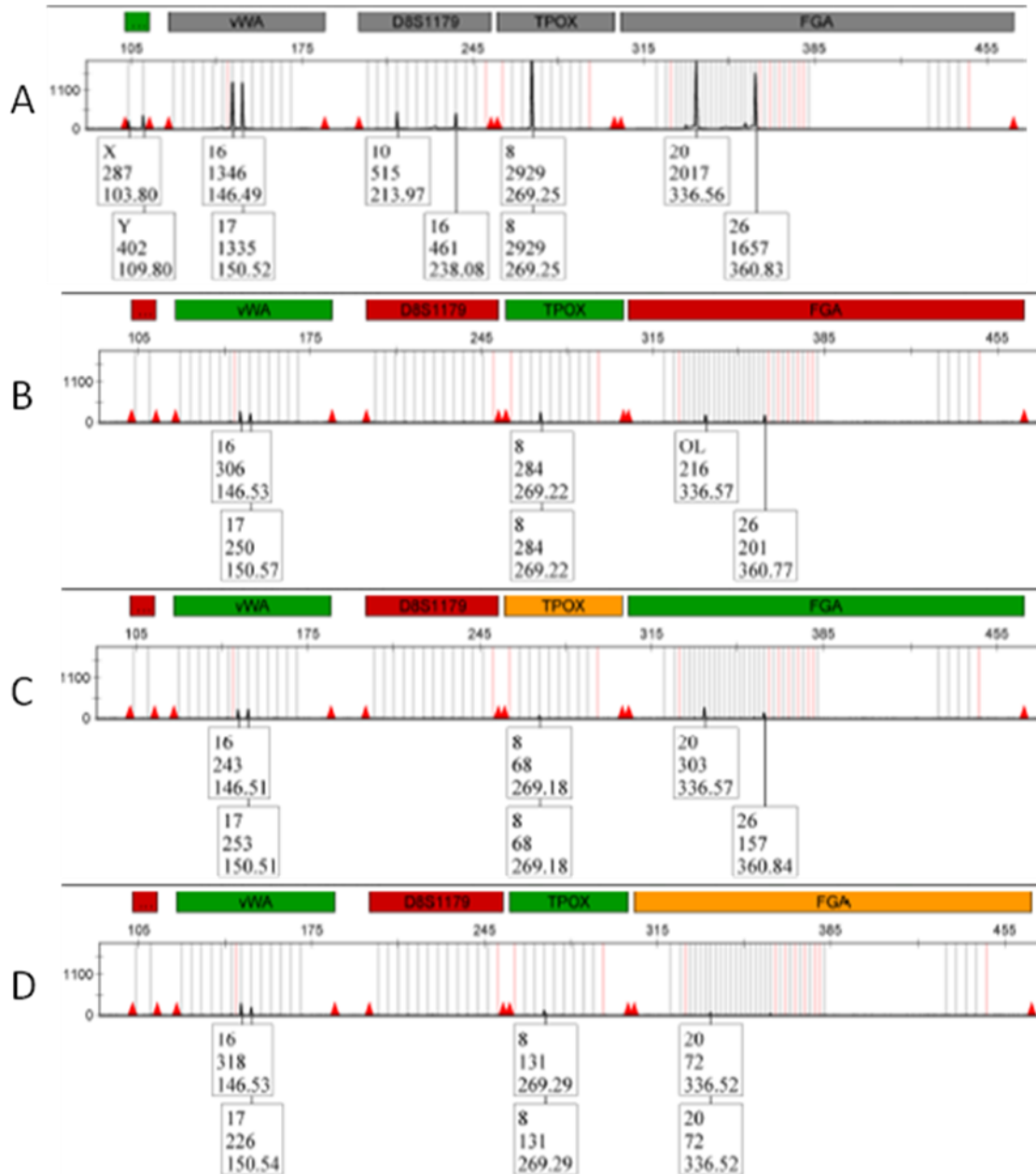


Figure 13. STR profile using DNA extracted from 25mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 17

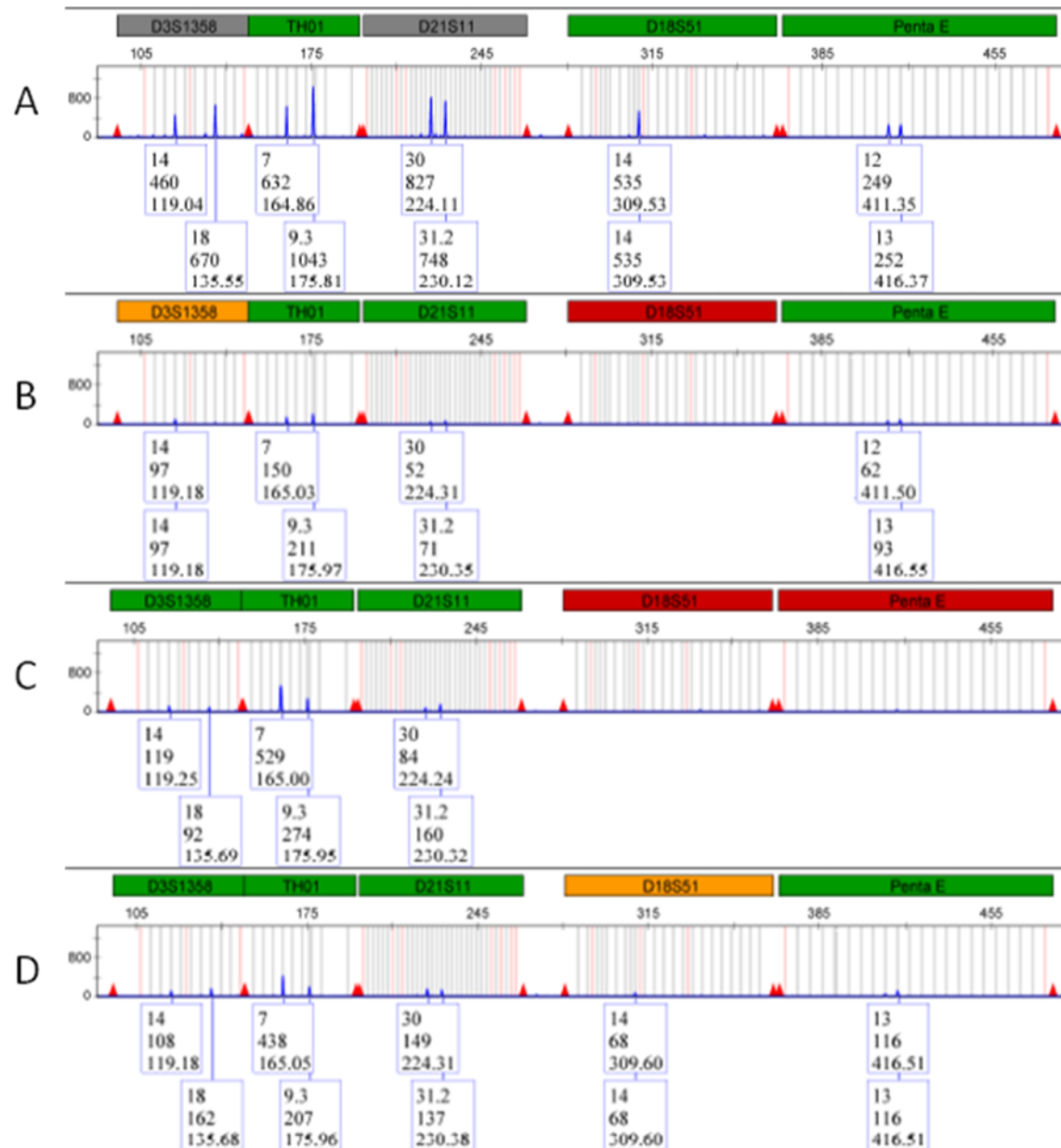


Figure 14. STR profile using DNA extracted from 50mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step.
Note: only 5 of the 16 loci are shown.

Appendix 18

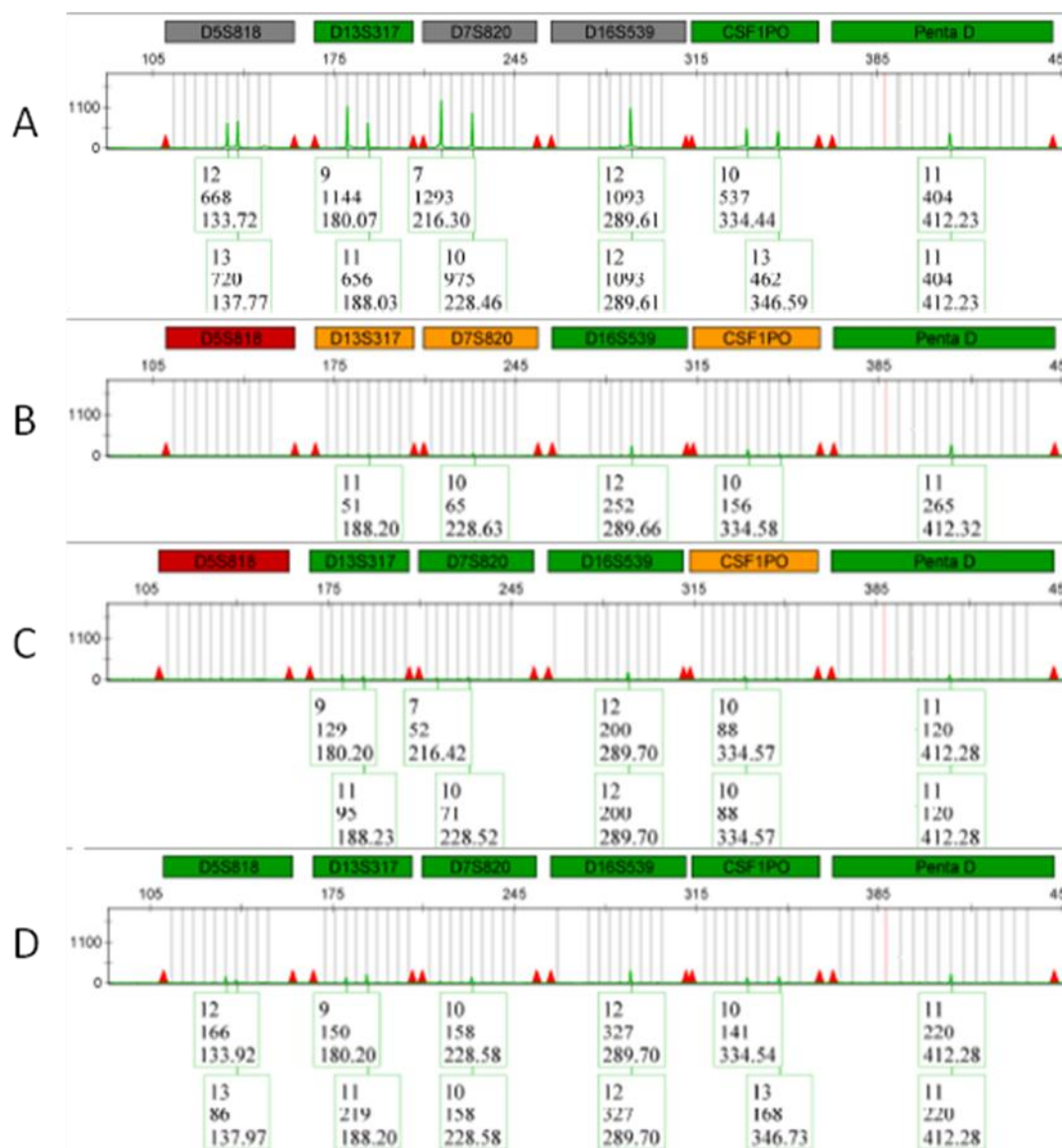


Figure 15. STR profile using DNA extracted from 50mg of the soil/seed mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20μL (B), 10μL (C) and 5μL (D) were used for the DNA binding step. Note: only 6 of the 16 loci are shown.

Appendix 19

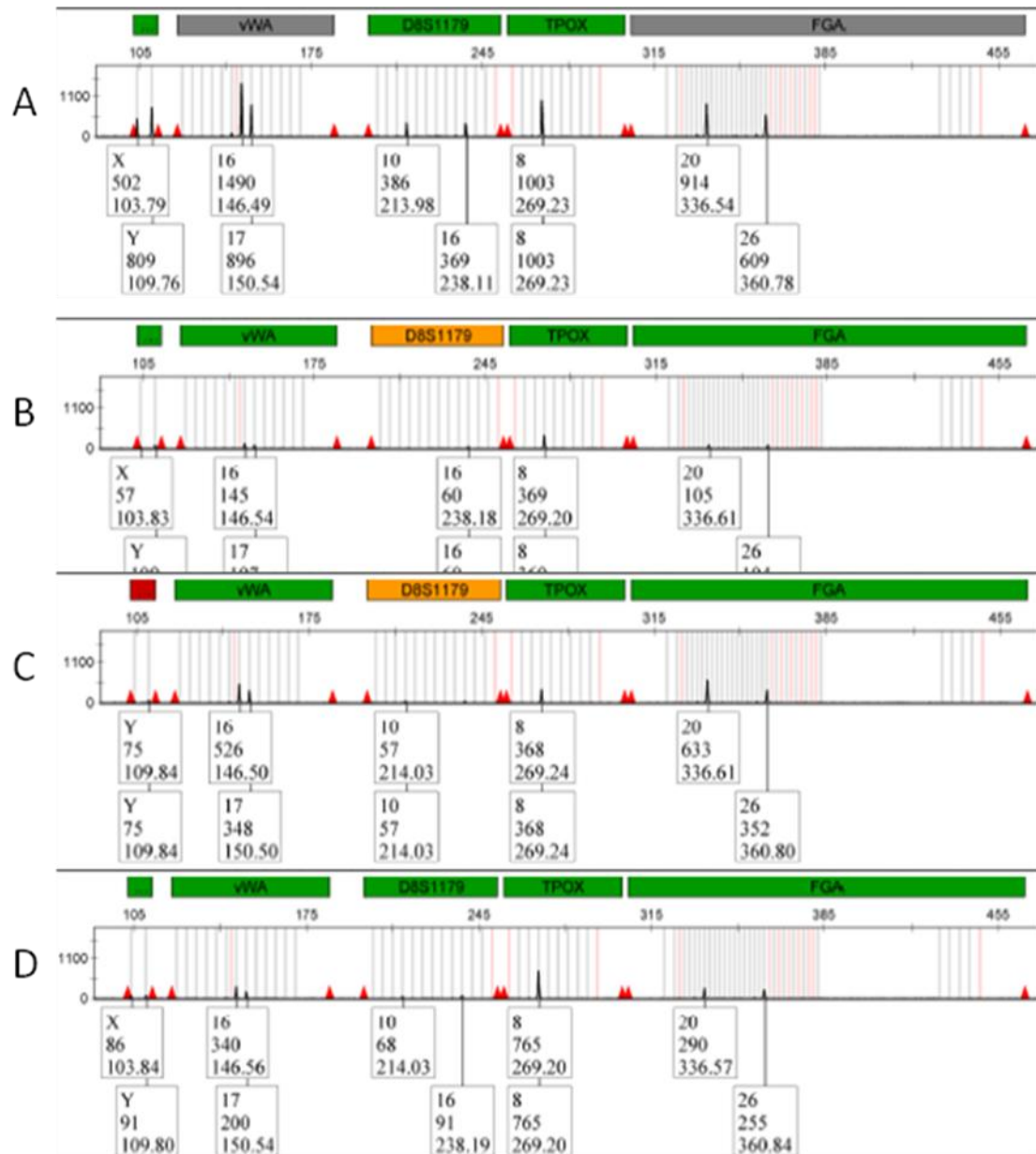


Figure 16. STR profile using DNA extracted from 50mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 20

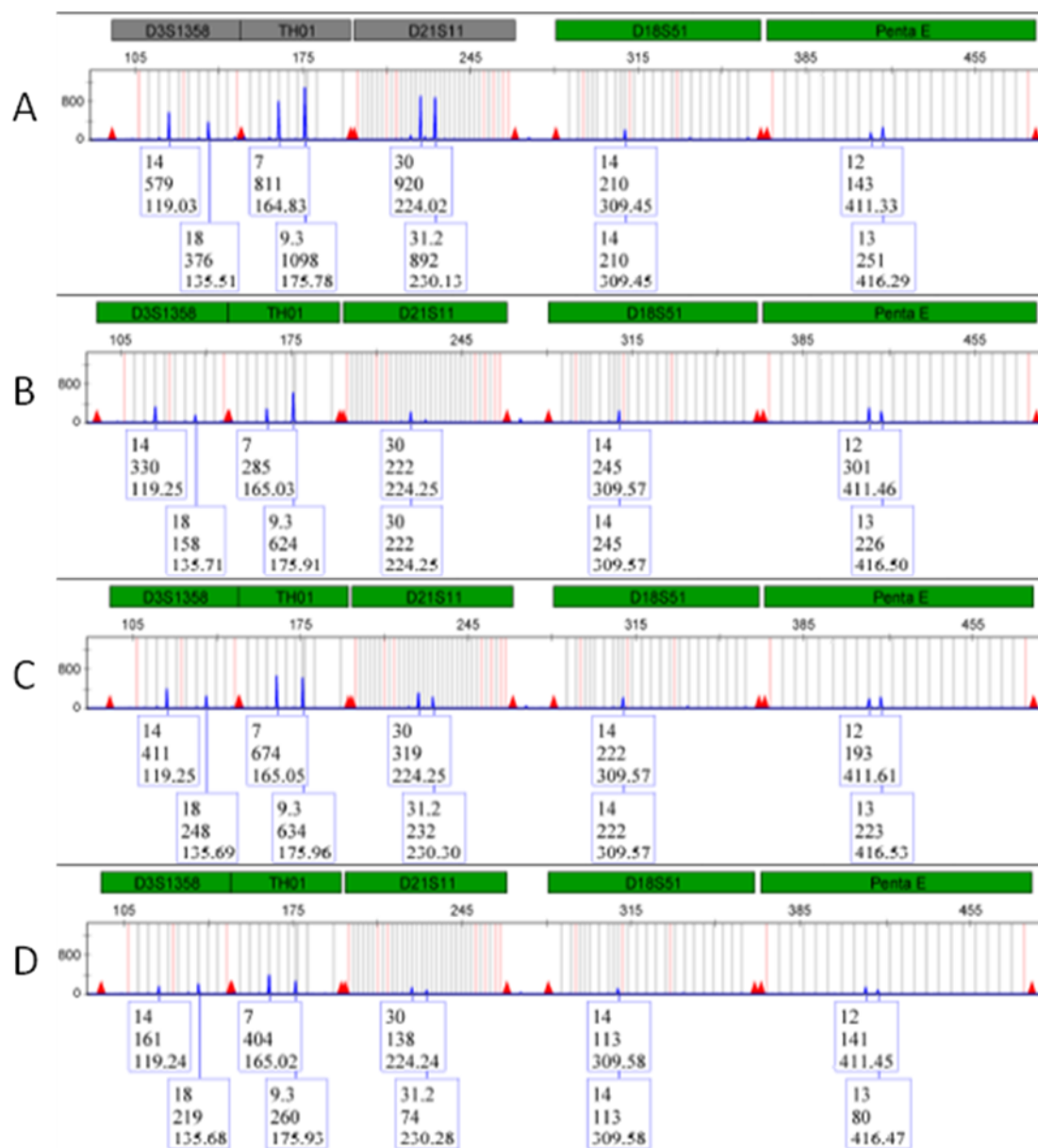


Figure 17. STR profile using DNA extracted from 100mg of the soil/seed mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 21

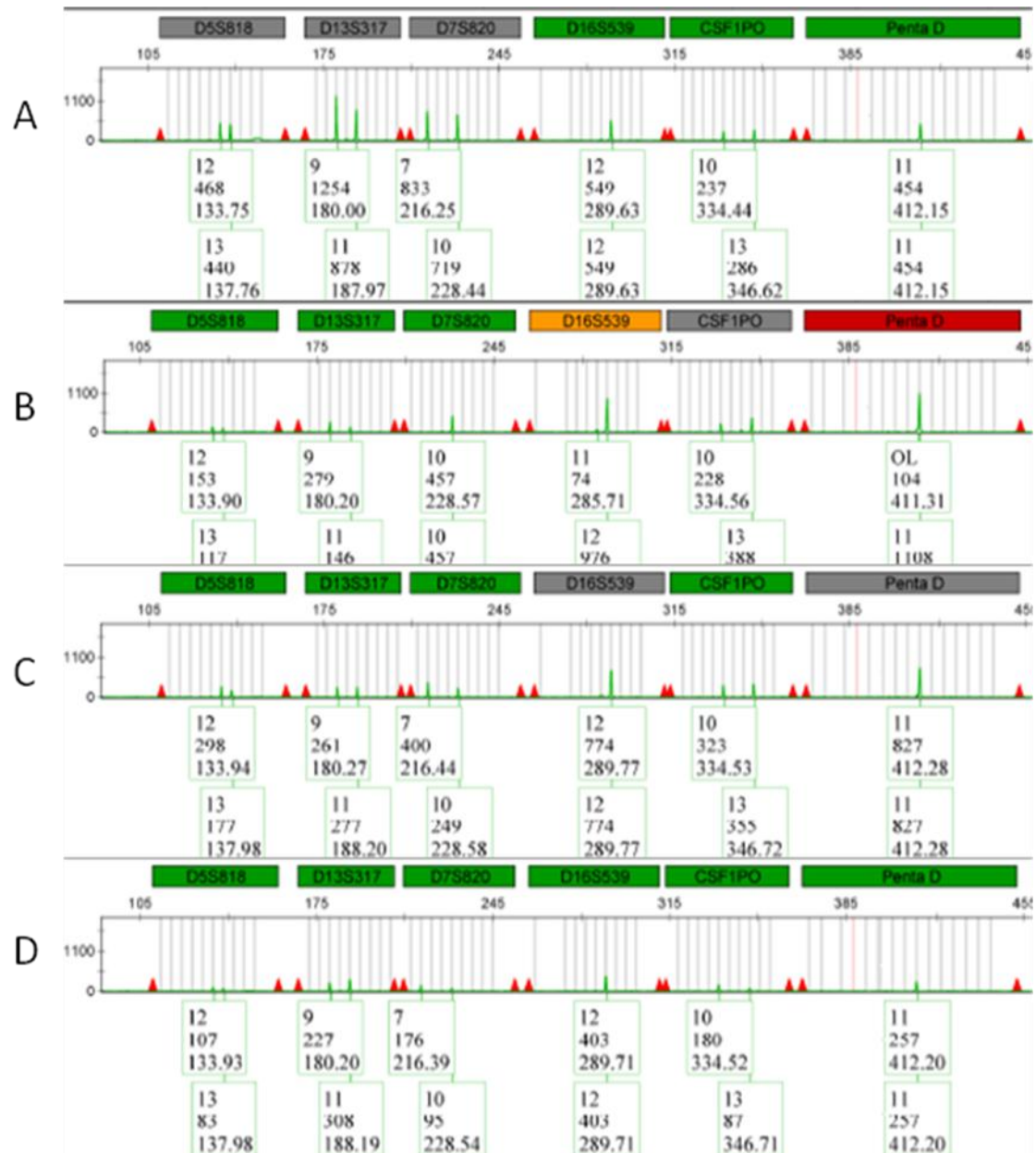


Figure 18. STR profile using DNA extracted from 100mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 6 of the 16 loci are shown.

Appendix 22

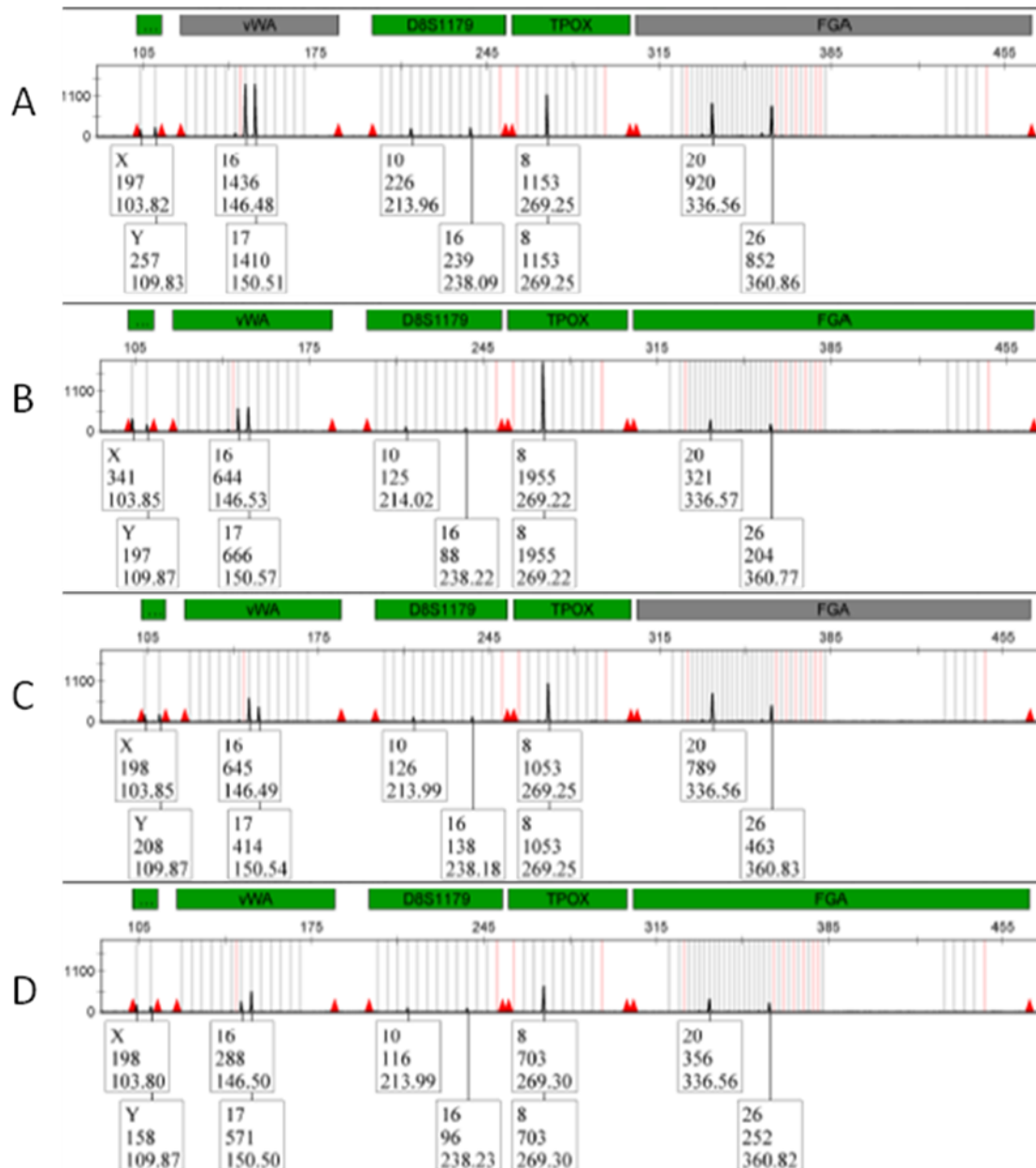


Figure 19. STR profile using DNA extracted from 100mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20 μ L (B), 10 μ L (C) and 5 μ L (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 23

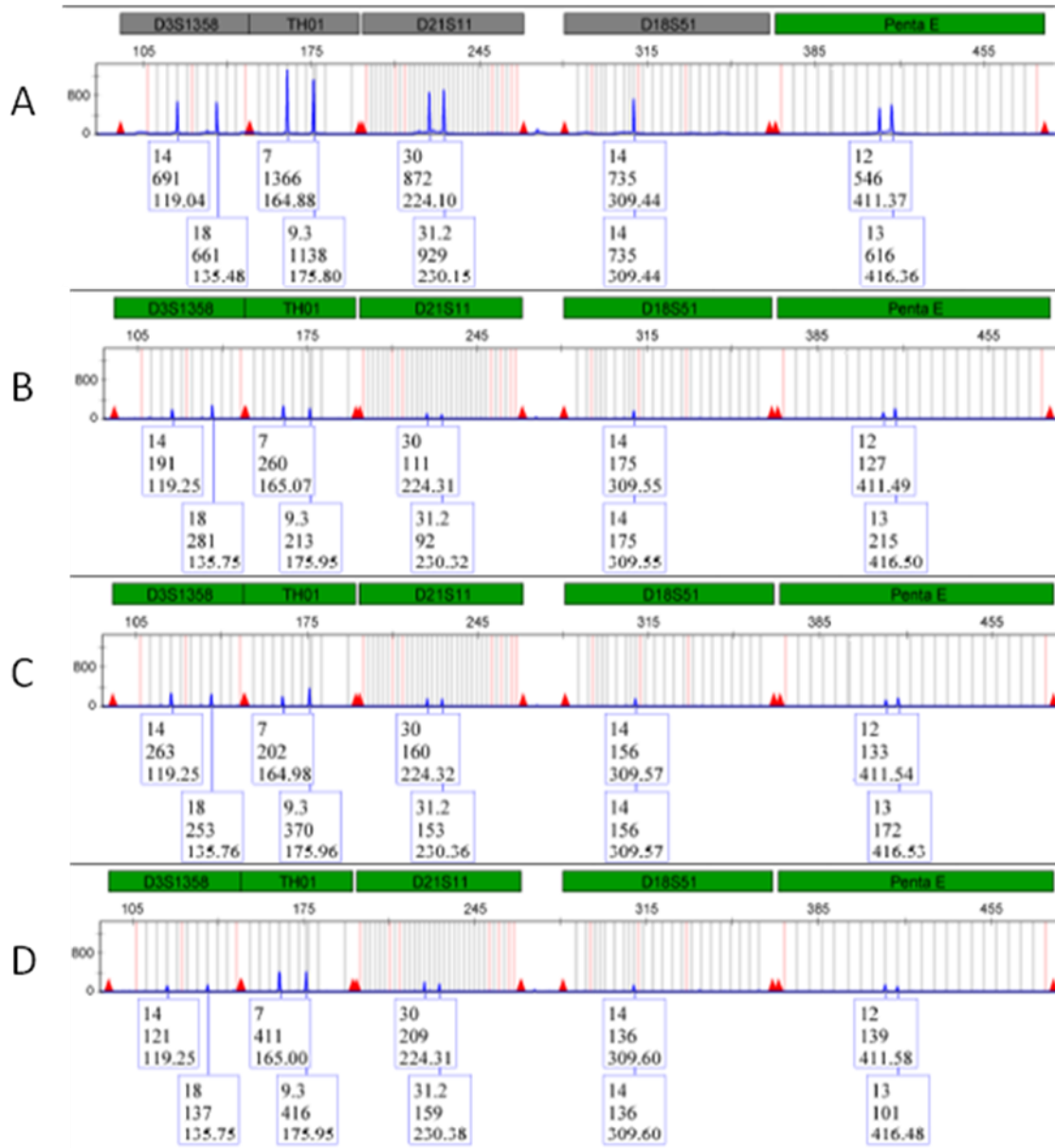


Figure 20. STR profile using DNA extracted from 125mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 24

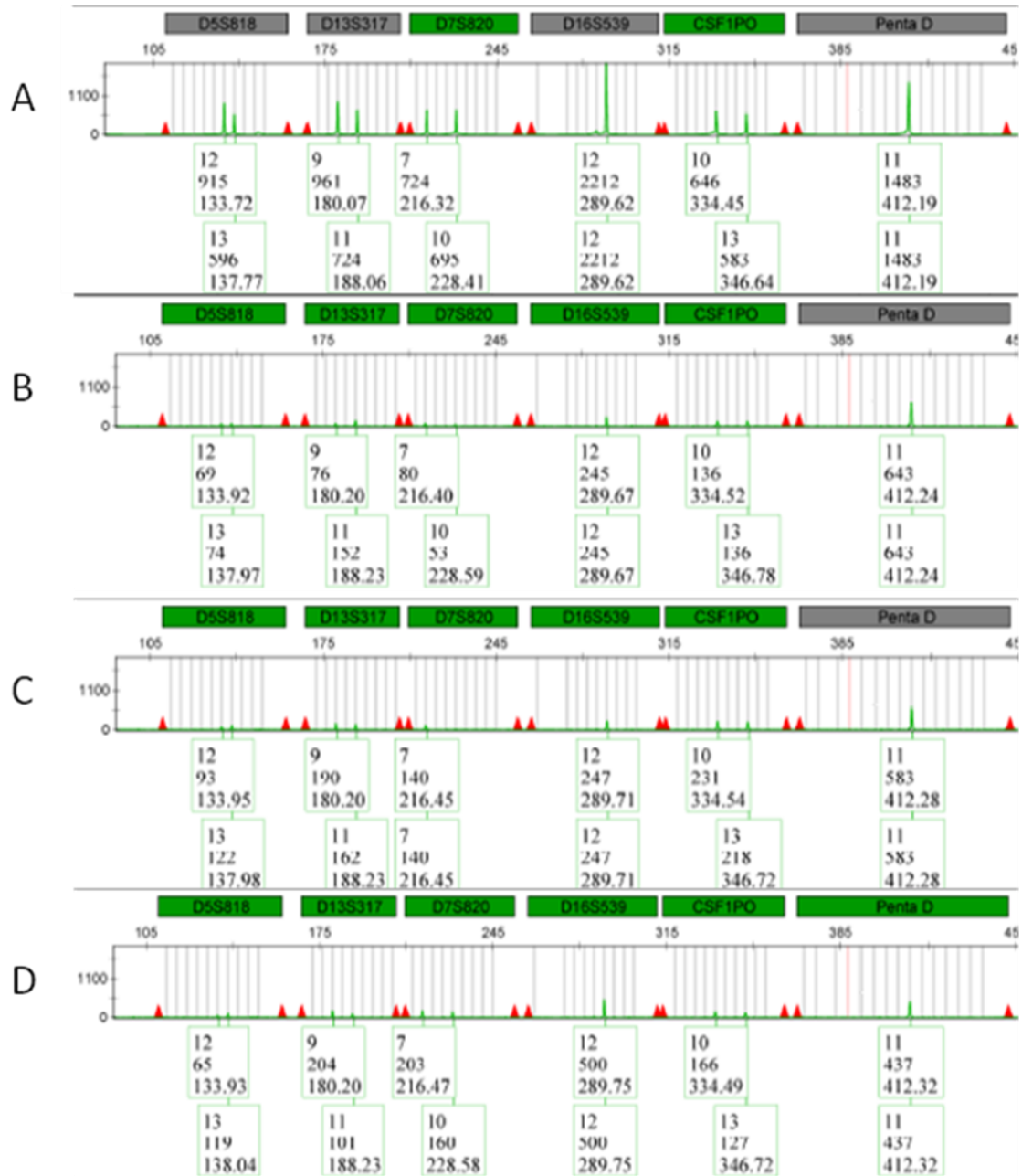


Figure 21. STR profile using DNA extracted from 125mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20μL (B), 10μL (C) and 5μL (D) were used for the DNA binding step. Note: only 6 of the 16 loci are shown.

Appendix 25

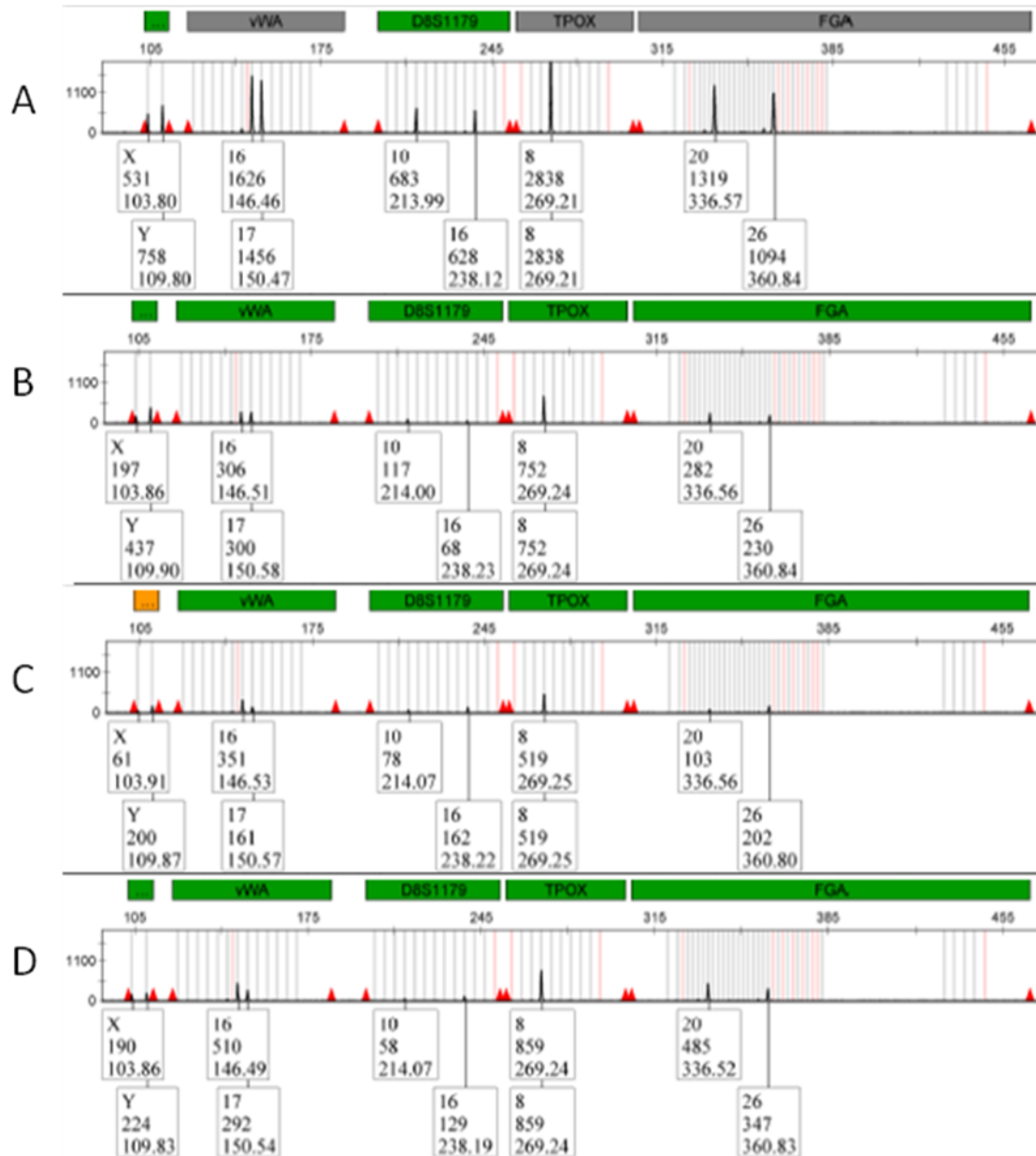


Figure 22. STR profile using DNA extracted from 125mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20 μ L (B), 10 μ L (C) and 5 μ L (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 26

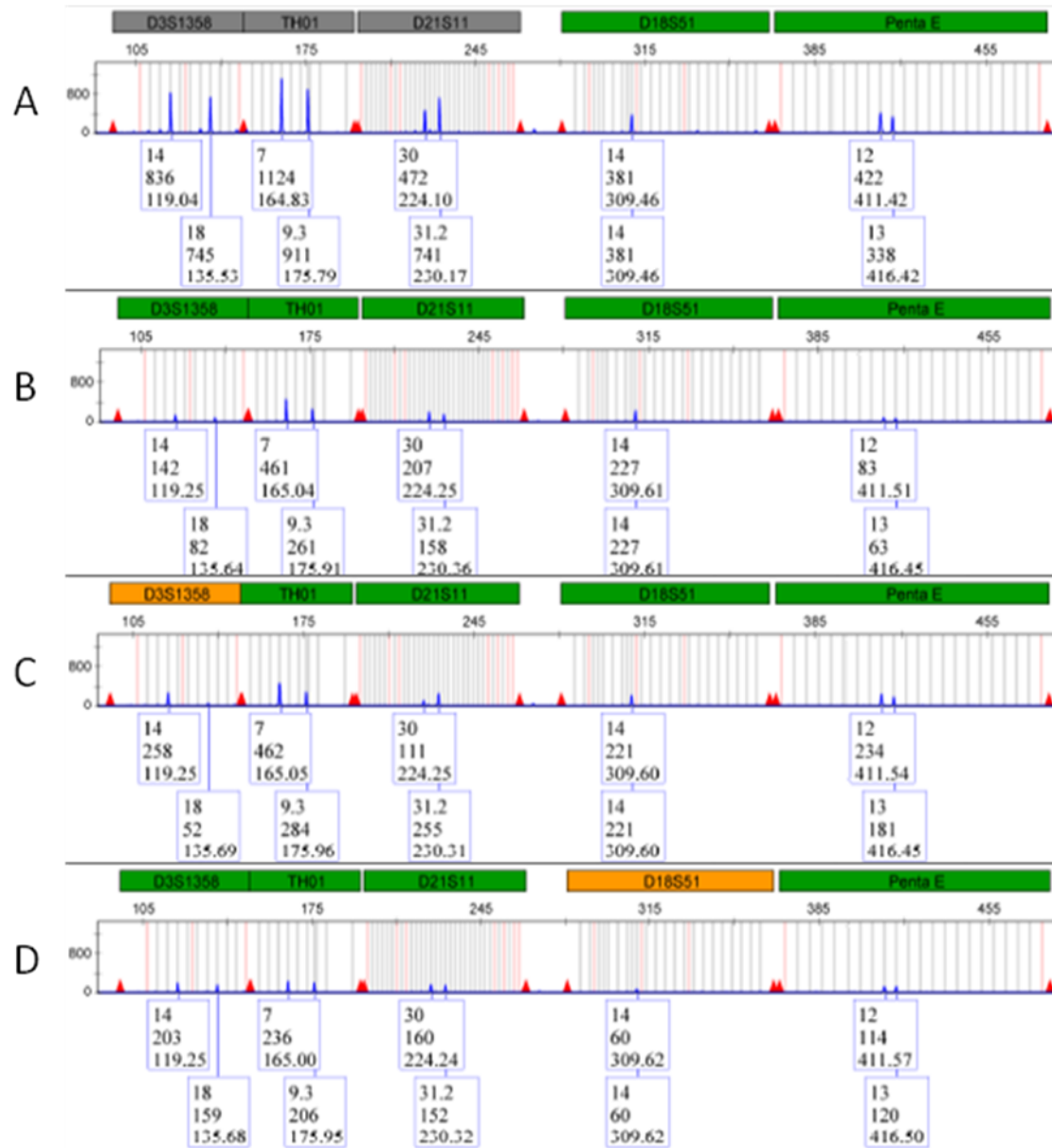


Figure 23. STR profile using DNA extracted from 250mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

Appendix 27

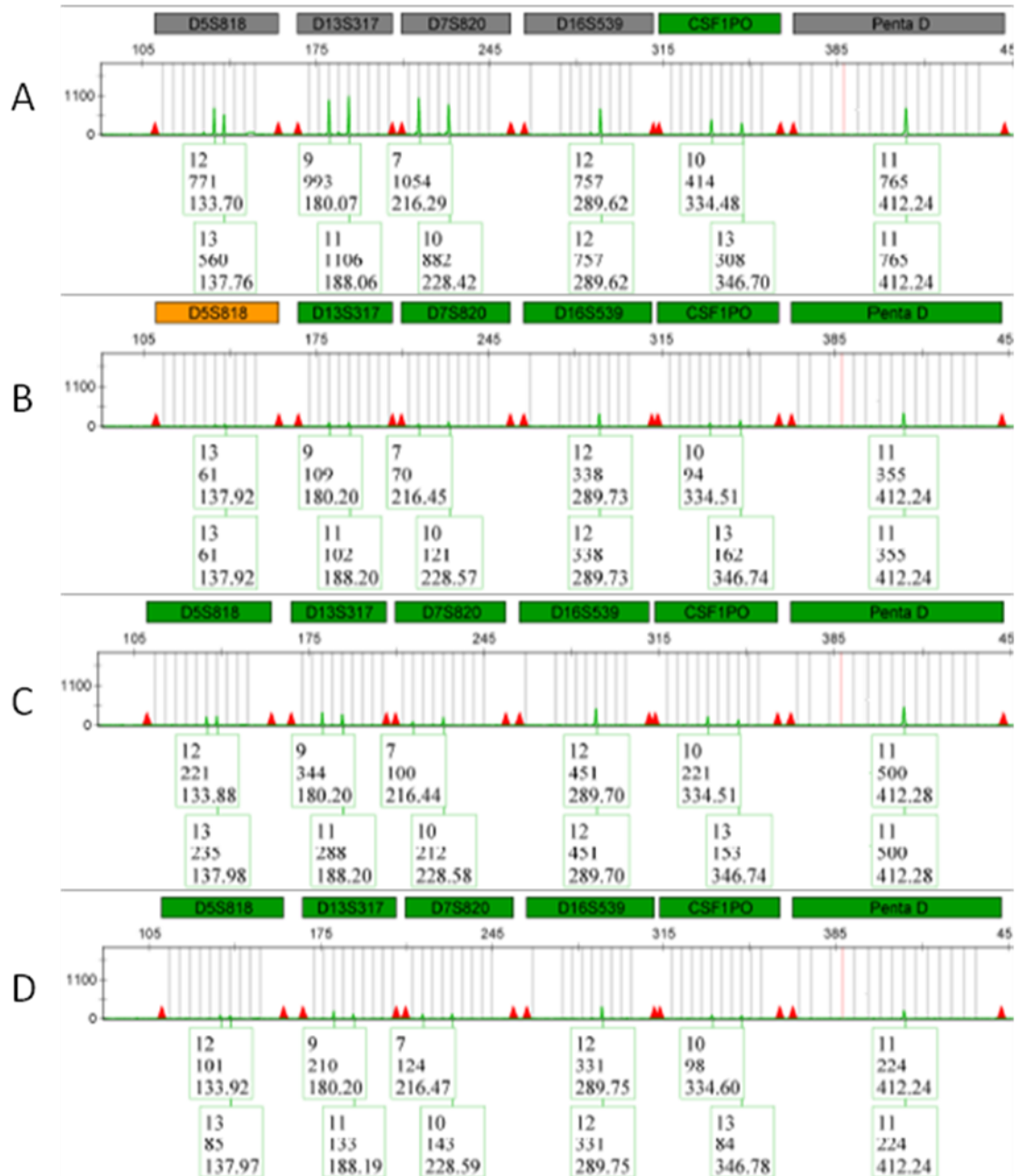


Figure 24. STR profile using DNA extracted from 250mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20µL (B), 10µL (C) and 5µL (D) were used for the DNA binding step. Note: only 6 of the 16 loci are shown.

Appendix 28

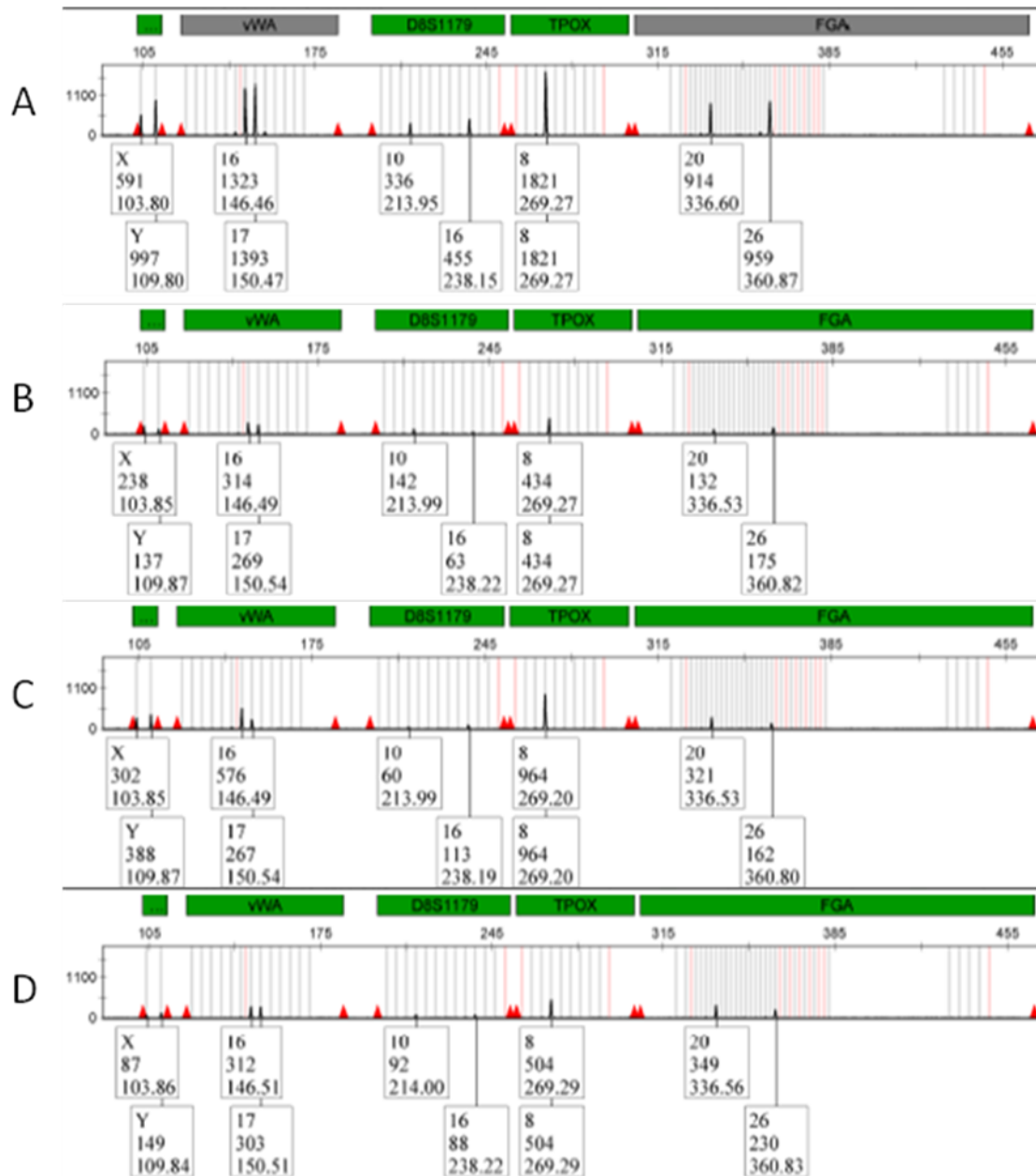


Figure 25. STR profile using DNA extracted from 250mg of the soil/semen mixture using PowerSoil (A) and SPION binding (three different amounts of magnetic beads i.e 20 μ L (B), 10 μ L (C) and 5 μ L (D) were used for the DNA binding step. Note: only 5 of the 16 loci are shown.

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